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(54) Title: CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES

(57) Abstract

A cellular immunogen is provided for immunizing a host against the effects of the product of a target proto-oncogene, where the overexpression of the target proto-oncogene is associated with a malignancy. The cellular immunogen comprises host cells which have been transfected with at least one transgene construct comprising a transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells. The transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene. The transgene may comprise, for example, wild-type or mutant retroviral oncogene DNA cognate to the target proto-oncogene; or wild-type or mutant proto-oncogene DNA of a species different from the host species. The cellular immunogen may be prepared from biopsied host cells, e.g. skin fibroblasts, which are stably or transiently transfected with the transgene construct containing the cognate transgene. The host cells transfected with the cognate transgene construct, are then returned to the body of the host to obtain expression of the cognate transgene in the host.

"CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES"

Cross-Reference to Related Application

Priority from U.S. provisional patent application No. 60/010,262, filed January 19, 1996 is claimed.

5   ## Field of the Invention

The invention relates to the field of cancer vaccination and immunotherapy.

Background of the Invention

A current goal of cancer research is the identification of host
10   factors that either predispose to tumor formation or serve to enhance tumor growth.

Genes that confer the ability to convert cells to a tumorigenic state are known as **oncogenes**. The transforming ability of a number of retroviruses has been localized in individual viral oncogenes (generally v-*onc*).
15   Cellular oncogenes (generally c-*onc*) present in many species are related to viral oncogenes. It is generally believed that retroviral oncogenes may represent escaped and/or partially metamorphosed cellular genes that are incorporated into the genomes of transmissible, infectious agents, the retroviruses.

Some c-*onc* genes intrinsically lack oncogenic properties, but may
20   be converted by mutation into oncogenes whose transforming activity reflects the acquisition of new properties, or loss of old properties. Amino acid

substitution can convert a cellular proto-oncogene into an oncogene. For example, each of the members of the c-*ras* proto-oncogene family (H-*ras*, N-*ras* and K-*ras*) can give rise to a transforming oncogene by a single base mutation.

5   Other c-*onc* genes may be functionally indistinguishable from the corresponding v-*onc*, but are oncogenic because they are expressed in much greater amounts or in inappropriate cell types. These oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized example of this type of proto-oncogene is c-*myc*. Changes in *MYC* protein sequence do not appear to be essential for

10   oncogenicity. Overexpression or altered regulation is responsible for the oncogenic phenotype. Activation of c-*myc* appears to stem from insertion of a retroviral genome within or near the c-*myc* gene, or translocation to a new environment. A common feature in the translocated loci is an increase in the level of c-*myc* expression.

15   Gene amplification provides another mechanism by which oncogene expression may be increased. Many tumor cell lines have visible regions of chromosomal amplification. For example, a 20-fold c-*myc* amplification has been observed in certain human leukemia and lung carcinoma lines. The related oncogene N-*myc* is five to one thousand fold amplified in

20   human neuroblastoma and retinoblastoma. In human acute myeloid leukemia and colon carcinoma lines, the proto-oncogene c-*myb* is amplified five to ten fold. While established cell lines are prone to amplify genes, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular oncogenes in many independent tumors of the same type, strengthens

25   the correlation between increased expression and tumor growth.

Immunity has been successfully induced against tumor formation by inoculation with DNA constructs containing v-*onc* genes, or by inoculation with v-*onc* proteins or peptides. A series of reports describe a form of "homologous" challenge in which an animal test subject is inoculated with either

30   v-*src* oncoprotein or DNA constructs containing the v-*src* gene. Protective immunity was induced against tumor formation by subsequent challenge with v-

src DNA or v-*src*-induced tumor cells. *See*, Kuzumaki *et al.*, *JNCI* (1988), 80:959-962; Wisner *et al.*, *J. Virol.* (1991), 65:7020-7024; Halpern *et al.*, *Virology* (1993), 197:480-484: Taylor *et al.*, *Virology* (1994), 205:569-573; Plachy *et al.*, *Immunogenetics* (1994), 40:257-265. A challenge is said to be "homologous" where reactivity to the product of a targeted gene is induced by immunization with the same gene, the corresponding gene product thereof, or fragment of the gene product. A challenge is "heterologous" where reactivity to the product of a targeted gene is induced by immunization with a different gene, gene product or fragment thereof.

WO 92/14756 (1992) describes synthetic peptides and oncoprotein fragments which are capable of eliciting T cellular immunity, for use in cancer vaccines. The peptides and fragments have a point mutation or translocation as compared to the corresponding fragment of the proto-oncogene. The aim is to induce immunoreactivity against the mutated proto-oncogene, not the wild-type proto-oncogene. WO 92/14756 thus relates to a form of homologous challenge.

EP 119,702 (1984) describes synthetic peptides having an amino acid sequence corresponding to a determinant of an oncoprotein encoded by an oncogenic virus, which determinant is vicinal to an active site of the oncoprotein. The active site is a region of the oncoprotein required for oncoprotein function. e.g., catalysis of phosphorylation. The peptides may be used to immunize hosts to elicit antibodies to the oncoprotein active site. EP 119,702 is thus directed to a form of homologous challenge.

The protein product encoded by a proto-oncogene constitutes a self antigen and, depending on the pattern of its endogenous expression, would be tolerogenic at the level of T cell recognition of the self peptides of this product. Thus, vaccination against cancers which derive from proto-oncogene overexpression is problematic.

Recent attempts have been made to induce immunity *in vitro* or *in vivo* to the product of the HER-2/*neu* proto-oncogene. The proto-oncogene encodes a 185-kDa transmembrane protein. The HER-2/*neu* proto-oncogene is overexpressed in certain cancers, most notably breast cancer. In each report

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discussed below, the immunogen selected to induce immunity comprised a purified peptide of the p185$^{HER-2/neu}$ protein, and not a cellular immunogen.

Disis *et al.*, *Cancer Res.* (1994) 54:16-20 identified several breast cancer patients with antibody immunity and CD4 + helper/inducer T-cell immunity responses to p185$^{HER-2/neu}$ protein. Antibodies to p185$^{HER-2/neu}$ were identified in eleven of twenty premenopausal breast cancer patients. It was assumed prior to this work that patients would be immunologically tolerant to HER-2/*neu* as a self-protein and that immunity would be difficult to generate.

Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076 constructed synthetic peptides identical to p185$^{HER-2/neu}$ protein segments with amino acid motifs similar to the published motif for HLA-A2.1-binding peptides. Out of four peptides synthesized, two were shown to elicit peptide-specific cytotoxic T-lymphocytes by primary *in vitro* immunization in a culture system using peripheral blood lymphocytes from a normal individual homozygous for HLA-A2. Thus, it was concluded that the p185$^{HER-2/neu}$ proto-oncogene protein contains immunogenic epitopes capable of generating human CD8$^+$ cytotoxic T-lymphocytes.

The cytotoxic T cells elicited in the latter report were not, however, shown to recognize tumor cells, but only targets that bound the synthesized peptides. Other work (Dahl *et al.*, *J. Immunol.* (1996), 157:239-246) has demonstrated that cytotoxic cells may recognize targets that bind peptide but fail to recognize targets that endogenously synthesize peptide. It is thus unclear whether the cytotoxic cells elicited by Disis *et al.* would be capable of recognizing tumor cells. In any event, no protection against tumor growth was demonstrated by Disis *et al.*

Peoples *et al.*, *Proc. Natl. Acad. Sci. USA* (1995), 92:432-436, report the identification of antigenic peptides presented on the surface of ovarian and breast cancer cells by HLA class I molecules and recognized by tumor-specific cytotoxic T lymphocytes. Both HLA-A2-restricted breast and ovarian tumor-specific cytotoxic T lymphocytes recognized shared antigenic peptides.

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T cells sensitized against a nine-amino acid sequence of one of the peptides demonstrated significant recognition of HLA-A2 HER2/*neu* tumors.

It remains unclear whether Peoples *et al.* have successfully attacked proto-oncogene-encoded self, as the immunizing peptide which is expressed in the tumor cells contained an isoleucine at position 2, whereas the peptide expressed in normal tissue contains valine residue at this position. Moreover, although stimulation of T cells occurred *in vitro*, this stimulation does not represent a true primary immune response insofar as the starting T cell population represented tumor infiltrating lymphocytes.

The research accounts of Disis *et al.* and Peoples *et al.* required a form of *in vitro* stimulation, either priming as described by Disis *et al.*, or restimulation as described by Peoples *et al.* The *in vitro* protocols of Disis *et al.* and Peoples *et al.* require a mutant cell line to aid in selection of the peptide which will serve to induce reactivity. Non-mutant, peptide antigen-presenting cells have their HLA class I molecules already loaded with endogenous peptides, a phenomenon which precludes exogenous loading from without. The value of the mutant lines is that they lack the TAP genes (encoding the transporters associated with antigen presentation). Class I binding of internally-derived peptides is significantly lowered, and "empty" class I molecules are present on the cell surface and available for binding of exogenously added peptides. This availability of peptide binding sites on membrane-bound class I allows examination of whether a given peptide will (i) even bind to class I, and (ii) function as a target in cytotoxic T cell assays. However, the need for a mutant cell line for deduction of candidate immunizing peptide sequences limits the usefulness of peptide-based immunization schemes.

Fendly *et al.*, *J. Biol. Response Modifiers* (1990), 9:449-455 present an account of a polypeptide-based immunotherapy. Purified polypeptide corresponding to the extracellular domain of the p185$^{HER-2/neu}$ protein was obtained from a transfected cell line. The purified peptide was employed in the immunization of guinea pigs. The immunized animals developed a cellular immune response, as monitored by delayed-type hypersensitivity. Antisera

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derived from immunized animals specifically inhibited the *in vitro* growth of human breast tumor cells overexpressing p185$^{HER-2/neu}$. There is no indication by Fendly *et al.* of induction of self versus non-self reactivity. It is likely that the guinea pigs were chiefly responding to non-self determinants (as defined in
5   terms of the guinea pig host) on the human polypeptide immunogen.

The use of peptides for immunization is of necessity limited to immunization with a single haplotype. There are approximately thirty HLA types in man. In each case of peptide immunization, one must be careful to select peptides which match the host HLA type. The selected peptide must be
10   immunogenic in the host and be capable of presentation to host immune system cells.

What is needed is an immunization method for immunizing humans and animals against self-encoded proto-oncogenes which are associated with the development of cancer, which dispenses with the need for isolating
15   immunogenic, HLA host-matched peptides for immunization.

Summary of the Invention

It is an object of the invention to induce reactivity to self-determinants of the product of an overexpressed proto-oncogene.

It is an object of the invention to provide for a form of therapy
20   or prophylaxis based upon the capacity to induce immune reactivity to proto--oncogene-encoded self as overexpressed in tumor cells.

It is an object of the invention to provide a cellular immunogen for use in immunization against self proto-oncogene determinants.

It is an object of the invention to provide for a method for
25   vaccinating a host against disease associated with the overexpression of a proto-oncogene.

These and other objects will be apparent from the following disclosure.

A method of vaccinating a host against disease associated with the
30   overexpression of a target proto-oncogene is provided. The method comprises:

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(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

According to one principal embodiment of the invention, the transgene comprises wild-type or mutant retroviral oncogene DNA. According to another principal embodiment of the invention, the transgene comprises wild-type or mutant proto-oncogene DNA of a species different from the host species. Where the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA, the mutant DNA is preferably nontransforming. The mutant DNA preferably comprises a deletion mutation in a region of the DNA which is essential for transformation. Preferably, the host cells are transfected with a plurality, most preferably at least five, different transgene constructs, each construct encoding a different deletion mutation.

In one preferred embodiment of the invention, the mutant DNA has at least about 75% homology, more preferably at least about 80% homology, most preferably at least about 90% homology, with the corresponding wild-type oncogene or proto-oncogene DNA.

The invention is further directed to a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which is associated with a cancer. The cellular

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immunogen comprises the host cells which have been transfected with at least one transgene construct, as described above.

The invention is also directed to a method of preparing the cellular immunogen, by (a) excising cells from the host, and (b) transfecting the
5   excised cells with at least one transgene construct, as described above.

The cells transfected with the transgene are preferably rendered non-dividing prior to return to the body of the host.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (*i.e.*, is identical, not strictly
10   evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human c-*myc* gene is the cognate gene to
15   the mouse c-*myc* gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode proteins which are functionally equivalent.

By "homology" is meant the degree of sequence similarity between two different amino acid sequences, as that degree of sequence
20   similarity is derived by the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably
25   linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

30   The word "transfection" is meant to have its ordinary meaning, that is, the introduction of foreign DNA into eukaryotic cells.

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By "transgene" is meant a foreign gene that is introduced into one or more host cells.

By "transgene construct" is meant DNA containing a transgene and additional regulatory DNA, such as promoter elements, necessary for the expression of the transgene in the host cells.

Description of the Figures

Fig. 1 is a plot of the mean tumor diameter over time following subcutaneous wing web inoculation of 1-day-old line TK (panel A) and line SC (panel B) chickens with 100 μg of tumorigenic plasmids pcsrc527 (—▲—), pVSRC-C1 (—●—) or pMVsrc (--■--). The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for pcsrc527, italics for pVSRC-C1, bold typeface for pMVsrc). Error bars (unless obscured by the symbol) indicate standard error.

Fig. 2 is a plot of the growth of challenge (wing web) tumors in test and control line TK chickens under conditions of (i) priming and homologous challenge with plasmid pcsrc527 (panel A: --△--, test; --▲--, control), or (ii) priming and homologous challenge with plasmid pVSRC-C1 (panel B: --○--, test; --●--, control). Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge diameter was computed as in Fig. 1. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). The statistical

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comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where $p < 0.05$. Error bars indicate standard error.

5   Fig. 3 is a plot of the growth of challenge (wing web) tumors in TK chickens under conditions of (i) priming with plasmid pVSRC-C1 and heterologous challenge with plasmid pcsrc527 (panel A: --△--, test; --▲--, control) or (ii) priming with pcsrc527 and heterologous challenge with pVSRC-C1 (panel B: --○--, test; --●--, control). Test chickens were primed at 1

10   day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as in Fig. 1. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for

15   test group). Statistical comparisons were made between test and control groups at a particular time point as described for Fig. 2. [*($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, $p < 0.05$. Error bars indicate standard error.

20   <u>Detailed Description of the Invention</u>

A vaccination strategy is provided to prevent development of cancers. The vaccination method may be carried out on a subject at risk for a particular cancer, but before the development of the cancer. The practice of the invention may serve for the immunoprevention of prevalent human cancers,

25   such as colon carcinoma, breast carcinoma, and various lymphomas whose progress is accompanied by the overexpression of a cellular proto-oncogene.

The vaccination strategy of the present invention relies on the induction of an immune response that targets tumor cells by virtue of the recognition of the proto-oncogene-specific antigenicity. The aim of the vaccine

30   protocol is to induce reactivity to self-determinants of an overexpressed proto-

oncogene product. The strategy exploits the structural relatedness between the product of the cellular proto-oncogene and that of the product of genes cognate to the target proto-oncogene. The cognate gene may comprise a wild-type or mutant cognate retroviral oncogene or a wild-type or mutant proto-oncogene

5   of a species different from the host species. The starting point of the vaccine strategy is the high degree of primary sequence homology that exists between the protein product of a targeted proto-oncogene and that of its cognate retroviral oncogene, or between the proto-oncogene product and the product of a cognate proto-oncogene from a different species. However, in contrast to

10   other proposed vaccine strategies, the present invention is not based on the immune recognition of a determinant defined by a cancer specific mutation.

For those tumors showing proto-oncogene overexpression, this sequence homology permits application of the following strategy, which can be employed either prophylactically or therapeutically under conditions of cell-

15   surface expression, or other forms of adjuvanicity, as chosen to enhance immunogenicity: (a) immunization of host biopsied cells with a DNA construct comprising a transgene cognate to the target proto-oncogene, which transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene; (b) return of the

20   transfected cells to the body of the host to obtain expression of the transgene in the host, and thus immunity against the proto-oncogene product. The invention relies on the targeting of a self-determinant found on an overexpressed or overabundant proto-oncogene-encoded product. The foreign peptide elements of the immunizing oncogene product will trigger peripheral lymphocytes

25   exhibiting a weak cross reactivity for the self peptides of the targeted proto-oncogene product. Although such self peptides would be present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

The immune strategy exploits the antigenicity of two alternative

30   types of determinants: (1) tumor-associated antigenic determinant(s) induced as a consequence of the activity of the oncogene product, e.g., an enzymatic

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modification of a cellular protein effected by the oncogene product, or (2) tumor

associated antigenic determinant(s) intrinsic to the oncogene-encoded product

itself. The difficulty in exploiting the first alternative by traditional means, i.e.,

antigen purification, is that at present little or no systematic information exists

5   bearing on the properties of an antigen that, though oncogene-induced, is not

oncogene-encoded. This situation makes purification of any such antigen

problematic. However, this problem is obviated from the outset by the present

invention which utilizes biopsied cells which, as transfected in culture by the

cognate retroviral oncogene, would express the relevant antigenicity.

10   In terms of exploiting the second alternative, that of an

antigenicity intrinsic to the proto-oncogene product, a relevant consideration is

that the protocol of immunization according to the present invention primes the

host to determinants of the oncogene product itself. A consequence of this

immunization is induction of T-cell reactivity to the divergent, i.e foreign,

15   peptide determinants of the retroviral oncogene product, i.e., those peptide

determinants that show sequence differences with the positionally homologous

determinants of the cellular proto-oncogene product. The induction of this

reactivity does not in itself have vaccine potential, since the foreign

determinants specific to the retroviral oncogene product are normally absent

20   from the cellular proto-oncogene product. Nevertheless, the foreign peptide

elements, notably those that differ by only a single amino acid from the

positionally homologous self peptides, trigger peripheral T-lymphocytes

exhibiting a weak cross-reactivity for the self peptides. Although such self

peptides are present in normal cells expressing the proto-oncogene, targeting of

25   the tumor cells is favored in view of their overexpression of the proto-oncogene.

It is possible that many tumor-associated and overexpressed

proto-oncogenes might possess mutations. In some cases, overexpression may

very well arise as a direct consequence of one or more of the mutations.

However, the present vaccination method does not have as its object the

30   deliberate targeting of non-self determinants generated by proto-oncogene

mutations. Unlike prior vaccination methods designed to target such mutation-

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driven non-self determinants, it is the aim of the present invention to induce reactivity for self-determinants in the overexpressed product of tumor associated and overexpressed proto-oncogenes.

Prior efforts attempting to elicit reactivity to proto-oncogene self
5   determinants have relied on *in vitro* protocols utilizing mutant cell lines to identify individual self peptide immunogens (Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076; Peoples *et al.*, *Proc. Natl. Acad. Sci USA* (1995), 92:432-436). According to the present invention, the host immune system is presented with the full array of naturally-derived class I binding peptides. The vaccine strategy
10   of the present invention obviates the need for any *a priori* assessment of the immunogenicity of individual peptides.

While the cellular immunogens of the invention display self peptides, non-self peptides would also be presented which may serve as more effective tolerance breakers. The value of a non-self, but closely related to self,
15   peptide is that it may more readily activate those T cells that have both a weak cross reactivity for the cognate self peptide and an activation threshold (determined by the tightness of binding to the T cell receptor) too high to be triggered by the self peptide. Moreover, cognate non-self is inductive of a good immune response, simply because it does in fact constitute nonself. The non-
20   self immune response is expected to predispose the induction of the inevitably weaker response to the self determinants on the same protein product, since the resultant cytokine release provides local help to initiate the weaker anti-self response.

As hereinafter exemplified in a model of *src*-oncogene-based
25   tumor formation, immunization with cells transfected with a transgene construct expressing the v-*src* oncogene product induces reactivity to the product of the c-*src* proto-oncogene, thereby conferring protection against the growth of tumors displaying overexpression of the c-*src* proto-oncogene.

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Target Proto-Oncogenes

According to the present invention, patients with a family history of a cancer characterized by the overexpression of a particular proto-oncogene are selected for immunization. Alternatively, patients whose tumors can be shown to overexpress the proto-oncogene are selected. Overexpression of a proto-oncogene may derive from an increase over a basal level of transcription. Overexpression may also derive from gene amplification, that is, an increase in gene copy number, coupled with a basal or elevated level of transcription. Proto-oncogene overexpression may be assayed by conventional probing techniques, such as described in *Molecular Cloning: A Laboratory Manual* J. Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1989. The level of target proto-oncogene expression may be determined by probing total cellular RNA from patient cells with a complementary probe for the relevant mRNA. Total RNA from the patient cells is fractionated in a glyoxal/agarose gel, transferred to nylon and hybridized to an appropriately labelled nucleic acid probe for the target mRNA. The number of relevant mRNA transcripts found in the patient cells is compared to that found in cells taken from the same tissue of a normal control subject.

As an alternative to measuring mRNA transcripts, the expression level of a target proto-oncogene may be assessed by assaying the amount of encoded protein which is formed. Western blotting is a standard protocol in routine use for the determination of protein levels. See *Molecular Cloning, supra*, Chapter 18, incorporated herein by reference. Accordingly, a cell lysate or other cell fraction containing protein is electrophoresed on a polyacrylamide gel, followed by protein transfer to nitrocellulose, and probing of the gel with an antibody specific for the protein in question. The probe step permits resolution of the desired protein from all other proteins in the starting mixture. The bound antibody may be prelabeled, *e.g.*, by a radioisotope such as ^{125}I, so as to permit its detection on the gel. Alternatively, a secondary reagent (usually an anti-immunoglobin or protein A) may be radiolabeled or covalently coupled

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to an enzyme such as horseradish peroxidase or alkaline phosphatase. The strength of the signal is proportional to the amount of the target protein. The strength of the signal is compared with the signal from a sample analyzed in the same manner, but taken from normal as opposed to tumor tissue.

5   A description of the methodology and use of Western blotting to determine the levels of the c-src-encoded protein pp60^{c-src} in adenomatous polyps (colonic epithelia) is provided by Cartwright et al., Proc. Natl. Acad. Sci. USA (1990), 87:558-562, the entire disclosure of which is incorporated herein by reference.

10   An at least about eight-fold increase in that gene's expression in the patient cells compared to expression in normal control cells from the same tissue would indicate candidacy for vaccination.

           Table 1 includes a partial list of representative proto-oncogenes, the overexpression of which has been associated with one or more malignancies.

15   Each listed proto-oncogene is a target proto-oncogene according to the present invention. The corresponding oncogene, of which the target proto-oncogene is the normal cellular homolog, is also identified. This list of target proto-oncogenes is intended to be representative, and not a complete list.

Table 1

20   ## Representative List of Target Proto-Oncogenes

Proto-Oncogene	Tumor	Comments/References
AKT-2	ovarian	v-Akt is the oncogene of the AKT8 virus, which induces lymphomas in mice.
		1. Bellacosa et al., (1995) Int. J. Cancer 64(4):280-5: Southern-blot analysis has shown AKT-2 amplification in 12.1% of ovarian carcinomas, while Northern bot analysis has

revealed overexpression of AKT-2 in 3 of 25 fresh ovarian carcinomas which were negative for AKT-2 amplification.

2. Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 89(19): 9267-71): Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.

AKT-2	pancreatic	Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93(8):3636-41: Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.
c-*erb*B-2	bladder	c-*Erb*B-2 is also known as HER2/*neu*. V-*erb*B is the oncogene of the avian erythroblastosis virus. 1. Underwood *et al.*, (1995) *Cancer Res.* 55(11):2422-30: Protein overexpression was observed in 45% of patients with non-recurrent disease and 50% of patients with recurrent disease; 9% of bladder tumors analyzed shoed gene amplification. 2. Coombs *et al.*, (1993) *Pathology* 169(1):35-42: c-*Erb*B-2 gene amplification was observed in 14% of bladder tumors analyzed. 3. Gardiner *et al.*, (1992) *Urolog. Res.* 20(2):17-20: Nineteen percent of primary transitional cell bladder carcinomas showed c-*erb*B-2 gene amplification.
c-*erb*B-2	breast	1. Molina *et al.*, (1966) *Anticancer Research* 16(4B):2295-300: Abnormal c-*erb*B-2 levels were found in 9.2% of patients with locoregional breast

carcinoma, and in 45.4% of patients with advanced disease. 2. DePotter *et al.*, (1995) *Virchows Arch.* 426(2):107-15: Overexpression of the oncoprotein is observed in about 20% of invasive duct cell carcinomas of the breast. 3. Bandyopadhyay *et al.*, (1994) *Acta Oncol.* 33(5):493-8: 35.4% of breast tumors showed c-*erb*B-2 overexpression; 17.4% showed gene amplification. 4. Fontana *et al.*, (1994) *Anticancer Res.* 14(5B):2099-104: 26% of samples showed c-*erb*B-2 amplification. 5. Press *et al.*, (1993) *Cancer Research* 53(20):4960-70: Amplified overexpression was identified in 38% of primary breast cancers. 6. Berns *et al.*, (1992) *Cancer Res.* 52(5):1107-13: 23% of primary breast cancer tissues exhibited amplification. 7. Delvenne *et al.*, (1992) *Eur. J. of Cancer* 28(2-3):700-5: c-*erb*B-2 mRNA was overexpressed in 34% of breast tumor samples. 8. Inglehart, (1990) *Cancer Res.* 50(20):6701-7: Two to thirty-two-fold gene amplification was found in multiple stages of tumor progression. 9. Slamon *et al.*, (1989) *Science* 244:707-12: A 28% incidence of amplification of c-*erb*B-2 was found in 189 primary breast cancers. 10. Kraus *et al.*, (1987) *EMBO J.* 6(3):605-10: Eight cell lines demonstrated c-*erb*B-2 mRNA levels ranging from 4 to 128-fold overexpression. 60% of all tumors analyzed showed elevated levels of c-*erb*B-2 mRNA.

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c-*erb*B-2	lung	1. Osaki *et al.*, (1995) *Chest* 108(1):157-62: Lung tissue overexpression of c-*erb*B-2 was discovered in 42.5% of samples. 2. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 64-fold increase in the amount of c-*erb*B-2 mRNA was observed; 33% of lung tumors showed overexpression of c-*erb*B-2.
c-*erb*B-2	ovarian	1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Abnormally high expression of c-*erb*B-2 was found in 31% of tumor samples. 2. Felip *et al.*, (1995) *Cancer* 75(8):2147-52: 21.7% of ovarian tumors showed overexpression of c-*erb*B-2. 3. Fan *et al.*, (1994) *Chin. Med. J.* 107(8):589-93: c-*erb*B-2 amplification was found in 30.8% (8 of 26) of human ovarian cancers. 4. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 24% of ovarian tumors showed c-*erb*B-2 overexpression. 5. Csokay *et al.*, (1993) *Eur. J. of Surg. Oncology* 19(6):593-9: c-*erb*B-2 amplification was found in 34% of fresh ovarian tumor samples. 6. McKenzie *et al.*, (1993) *Cancer* 71(12):3942-5: 30% of ovarian tumor samples indicated c-*erb*B-2 overexpression. 7. Hung *et al.*, (1992) *Cancer Letters* 61(2):95-103: A 100-fold c-*erb*B-2 overexpression was discovered in one human cell line. Two to four-fold amplification was also discovered.
MDM-2	leukemia	*MDM-2* is the murine double minute-2 oncogene. 1. Bueso-Ramos *et al.*, (1993) *Blood* 82(9):2617-

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23:   53% of cases showed overexpression of *MDM*-2 mRNA.   The level of *MDM*-2 mRNA overexpression in some cases of leukemias was comparable to that observed in some sarcomas, which demonstrate more than 50-fold *MDM*-2 gene amplification.   No evidence of gene amplification was observed.   2. Watanabe *et al.*, (1994) *Blood* 84(9):3158-65:   28% of patients with B-cell chronic lymphocytic leukemia or non-Hodgkin's lymphoma had 10-fold higher levels of *MDM*-2 gene expression. *MDM*-2 overexpression was found more frequently in patients at advanced clinical stages.

c-*myb*   colon

V-*myb*   is   the   oncogene   of   the   avian myeloblastoma virus. 1. Ramsay *et al.*, (1992) *Cell Growth and Diff.* 3(10):723-30: c-*myb* levels were always higher in colon cancer samples than normal tissue. 2. Alitalo *et al.*, (1984) *Proc. Natl. Acad. Sci.* 81(14):4534-8:   c-*myb* levels were always higher in colon cancer samples than normal tissue.

c-*myc*   breast

V-*myc* is the oncogene of the avian myelocytoma virus.   1.   Lonn *et al.*, (1995) *Cancer* 75(11):2681-7: Amplification of c-*myb* occurs in 16% of patients with breast cancer. 2. Hehir *et al.*, (1993) *J. of Surg. Oncology* 54(4):207-9: c-*myc* overexpression was found in 60% of breast carcinoma samples. 3. Kreipe *et al.*, (1993) *Cancer Research* 53(8):1956-61: Amplification of

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c-*myc* was found in 52.6% of samples that displayed a Ki-S1 labelling index exceeding 30%. 4. Watson *et al.*, (1993) *J. Nat. Cancer Inst.* 85(11):902-7: Amplification of c-*myc* occurs in up to 20 - 30% of breast cancers. 5. Berns *et al.*, (1992) *Cancer Research* 52(5):1107-13: Amplification was found in 20% of primary breast cancer patients; the range was 3-14 gene copies. 6. Watanabe *et al.*, (1992) *Cancer Research* 52(19):5178-82: Expression of c-*myc* was increased by 10-fold.

c-*myc*   gastric/   1. Rigas, (1990) *Clin. Gastroent.* 12(5):494-9:
          colorectal   Overexpression of c-*myc* is found in 80 of colon cancers. 2. Erisman *et al.*, (1988) *Oncogene* 2(4):367-78: Adenocarcinoma cell lines express 5-10-fold elevated levels of c-*myc* mRNA. Eight to thirty-seven-fold higher levels of c-*myc* protein was found in tumor cell lines compared to normal cells. 3. Sikora *et al.*, (1987) *Cancer* 59(7):1289-95: Up to 32-fold overexpression of c-*myc* mRNA was observed in 12 to 15 tumors. 4. Tsuboi *et al.*, (1987) *Biochem. and Biophys. Res. Comm.* 146(2):705-10: Gastric Cancer: A 2-3-fold overexpression was observed in gastric cancer. A 2-10-fold overexpression was observed in colorectal cancer.

c-*myc*   lung   1. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 57-fold increase in c-myc mRNA levels was observed. 23% of samples indicated strong

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expression of c-myc. 2. Kato *et al.*, (1993) *Jap. J. of Cancer Res.* 84(4):355-9: Liver tissue metastases from human small cell lung carcinoma revealed 30-fold amplification of c-*myc*.

| 5 | c-*myc* | naso-pharn-geal | Porter *et al.*, (1994) *Acta Oto-Laryng.* 114(1): 1105-9: 22% of samples showed intense staining for c-*myc*. |

| | c-*myc* | ovarian | 1. Bian *et al.*, (1995) *Chin. J. of Ob. Gyn.* 30(7):406-9: 50% of samples showed amplification of c-*myc*. 2. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: 26% of samples exhibited c-*myc* amplification. 3. van Dam *et al.*, (1994) *J. Clin. Path.* 47(10):914-9: Overexpression of c-*myc* was found in 35% of ovarian carcinomas. 4. Xin *et al.*, (1993) *Chin. J. of Ob. Gyn.* 28(7):405-7: 54.5% of samples showed amplification of c-*myc*. 5. Tashiro *et al.*, (1992) *Int. J. of Cancer* 50(5):828-33: Overexpression was found in 63.5% of all serous adenocarcinoma tissues and 37.3% of all ovarian carcinoma tissues. Significant overexpression of c-*myc* was observed at Stage III compared with other stages. |

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| | c-*myc* | prostate | Nag *et al.*, (1989) *Prostate* 15(2):115-22: A 10-fold amplification of c-*myc* was observed. Fifty-fold higher levels of mRNA transcripts of c-*myc* were found. |

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c-*ras*	lung	*Ras* oncogenes were first recognized as the transforming genes of Harvey and Kirsten murine sarcoma viruses. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: a 13-fold increase in overexpression of c-Ki-*ras* was observed. 18% of tumors displayed strong overexpression of c-Ki-*ras*.
c-*ras*	ovarian	1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Higher levels of *ras* protein than in normal or benign ovarian tumors were found in 45% of tumor samples. 2. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 20% of ovarian tumors exhibited c-*ras* overexpression. The levels of expression of c-*ras* were much higher in tumors of patients with recurrent or persistent disease after chemotherapy, than in the tumors of patients at initial presentation.
c-*src*	breast	V-*src* is the oncogene of the Rous sarcoma virus, which induces sarcomas in chickens. Muthuswamy *et al.*, (1994) *Mol. and Cell. Biol* 14(1):735-43: c-*erb*B-2-induced mammary tumors possessed 6-8-fold higher c-*src* kinase activity than adjacent epithelium.
c-*src*	colon/ colorectal	1. Cartwright *et al.*, (1994) *J. of Clin. Invest.* 93(2):509-15: c-*src* activity is 6-10-fold higher in mildly dysplastic ulcerative colitis (a chromic inflammatory disease of the colon with a high on incidence of colon cancer) than in non-dysplastic

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epithelia. This data suggests that activation of c-*src* is an early event in the genesis of UC colon cancer. 2. Talamonti *et al.*, (1993) *J. of Clin. Invest.* 91(1):53-60: High level of c-*src* activity from colorectal cancer is found in liver metastases. 3. Termuhlen *et al.*, (1993) *J. of Surg. Res.* 54(4):293-8: Colon carcinoma metastases to the liver had significantly increased activity of c-*src* with an average 2.2-fold increase. Extrahepatic colorectal metastases demonstrated an average 12.7-fold increase in c-*src* activity over normal mucosa.

c-*yes*	colon

V-*yes* is the oncogene of two avian sarcoma viruses, Esh sarcoma virus and Y73. 1. Pena *et al.*, (1995) *Gastroent.* 108(1):117-24: Twelve to fourteen-fold higher expression of c-*yes* was found in colonic transforming oncogene adenomas compared to normal mucosa. Activity of c-*yes* was elevated in adenomas that are at greatest risk for developing cancer. 2. Park *et al.*, (1993) *Oncogene* 8(10):2627-35: A ten to 20-fold higher than normal activity of c-*yes* was observed in 3 out of 5 colon carcinoma cell lines. A 5-fold higher than normal activity was found in 10 out of 21 primary colon cancers, compared to normal colonic cells.

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Selection of Cognate Transgene for Preparation of Cellular Immunogen

According to the present invention, a transgene construct is
engineered comprising a transgene which is cognate to the target proto-oncogene
(hereinafter "cognate transgene" or "CTG"). The transgene is selected such that
5   it encodes a gene product which induces host immunoreactivity to host self-
determinants of the product of the target proto-oncogene. The transgene should
be expressed to very high levels in the transfectants. Thus, the construct should
contain a strong promoter.

The product encoded by the cognate gene must have a high
10   degree of sequence homology with the product of the target proto-oncogene, but
also must display some amino acid differences with the target proto-oncogene
product. Thus, there must be a subset of one or more amino acid differences
between the target proto-oncogene and its cognate in order to provide
immunogenic stimulus. Two classes of genes that satisfy these criteria are
15   retroviral oncogenes and xenogenic proto-oncogenes. The word "xenogenic"
is intended to have its normal biological meaning, that is, a property or
characteristic referring or relating to a different species. Thus, a xenogenic
proto-oncogene is meant to include the a homologous proto-oncogene of a
species other than the host organism species. It may be appreciated that in the
20   case of a target proto-oncogene, e.g. MDM2, for which no retroviral homolog
is yet known, a xenogenic homologue is advantageously utilized as the source
of the DNA for the cognate transgene.

In principle, a more effective immunogenic stimulus would
depend on the particular sequence, and not on the distinction between a
25   retroviral oncogene and a xenogenic proto-oncogene in terms of their relative
transforming capacity. Thus, in certain cases, a retroviral oncogene may be
better at providing a tolerance-breaking immunogenic stimulus, and in other
cases, a xenogenic proto-oncogene may be more effective.

The retroviral oncogene or xenogenic proto-oncogene DNA
30   forming the CTG may comprise the wild type oncogene or proto-oncogene
DNA. More preferably, a mutant DNA is utilized, which is engineered so as

to be non-transforming in the host. The DNA is mutated to include one or more nucleotide insertions, deletions or substitutions which will encode an oncogene product which is nontransforming in the host, but retains the requisite degree of sequence homology with respect to the target proto-oncogene. A

5   cognate transgene deletion mutant (hereinafter "dCTG") is preferred.

A protein sequence is generally considered "cognate" with respect to the target proto-oncogene-encoded protein if it is evolutionarily and functionally related between species. A more precise view of cognation is based upon the following sequence comparison carried out utilizing the FASTA

10   program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference. Cognation is attained upon satisfying two criteria imposed by FASTA; (i) alignment of segments corresponding to at least 75% of the target proto-oncogene's encoded amino acid sequence; (ii) at least 80% amino acid identity

15   within the aligned sequences. The segments of the target proto-oncogene protein sequence and protein test sequence satisfying the two criteria are referred to as "homology regions". Accordingly, at least 75% of the target proto-oncogene protein sequence is alignable with the test sequence. The alignable segments or homology regions may, however, represent less than 75%

20   of the total test polypeptide chain for the case of test sequences that may significantly exceed the target proto-oncogene protein in length.

One skilled in the art, armed with the FASTA program, may survey existing sequence data bases (either protein sequences or DNA sequences, insofar as the amino acid sequence is determined by FASTA for all

25   reading frames) for test sequences which are cognate with respect to the target proto-oncogene. At the same time, one can isolate and then sequence what are very likely to be cognate test sequences (*e.g.* feline MDM-2, as likely to be cognate to human MDM-2) and use FASTA to verify the presumed cognation. according to the criteria set above. One may obtain the sequences of

30   presumptive cognate proto-oncogenes from a large number of mammalian

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sequences and screen these sequences with FASTA according to the aforesaid formulation of cognation.

Because the product encoded by a CTG differs at a small number of amino acid positions from the product encoded by the target proto-oncogene, an immunogenic stimulus is provided that (i) is directed against the foreign protein and (ii) with a lower probability, induce an anti-self response. The CTG is selected such that the gene product will yield the greatest immunogenic stimulus to induce anti-self reactivity. Provided that overall sequence homology (preferably greater than about 75%) is maintained, the presence of scattered amino acid differences is desired, since any one residue would likely have a relatively low probability of inducing self-reactivity. Moreover, the greatest number of residue differences would be advantageous, consistent with maintaining the requisite degree of general sequence homology.

The selection of amino acid modifications for the CTG may be facilitated by resort to available computer-based models used to identify immunogenic peptide fragments of polypeptides. These models could be employed to select CTGs which would possess the maximum number of immunogenic peptides for a given HLA haplotype.

Screening Procedure for CTG Selection

Notwithstanding the availability of computer-based algorithms which have some predictive value, it is desirable to design CTGs with resort to a screening procedure based on an actual experimental assay that can be HLA-haplotype specific. Accordingly, cells are biopsied from a normal volunteer of particular haplotype. The cells are transfected with a CTG construct, preferably a dCTG construct, satisfying the criteria set for cognition. More preferably, the cells are transfected with multiple dCTGs, preferably at least five dCTGs, satisfying the criteria for cognition. The at least five dCTGs are selected to display amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The transfected cells are then used to immunize the volunteer in accordance with the immunization method of the

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present invention. After immunization, the human subject is tested in a standard delayed hypersensitivity (DH) reaction with 10^4-10^6 irradiated, autologous fibroblasts, as transfected with the same dCTG (or series of dCTGs) as used for the immunizing preparation. A positive DH reaction (induration) would verify

5   the induction of reactivity. The induction of reactivity in this assay is readily demonstrable because of the priming to the non-self determinants on the dCTG-encoded protein and the readout in the DH reaction of the same nonself determinants. Once DH reactivity is demonstrated in a DH reaction that directly tests the antigenicity of the non-self determinants encoded by the dCTG

10   (i.e., priming with a non-self construct, DH testing with the same non-self construct), the subject can be then tested in a DH reaction based on testing with the autologous cells transfected with a dCTG derived from the human proto-oncogene itself (i.e., priming with a non-self construct, testing with the human self construct). Testing of a battery of human volunteers will lead to a

15   catalogue of HLA-matched dCTGs, such that, for individuals of the same HLA haplotype, the use of the particular dCTG would be inductive of reactivity to proto-oncogene-encoded self. Different CTGs may thus be tested so as to correlate maximal secondary stimulation with a particular HLA haplotype.

At the same time, this procedure may be used with patients

20   undergoing tumor resection (if post-operative immuno-suppressive protocols are not mandatory), such that prior to resection, a course of immunization would have been initiated, the endpoint of which would represent the development of a DH reaction.

Any given amino acid difference between the CTG-encoded

25   product and the proto-oncogene-encoded product has a low probability of being a "tolerance-breaker". Thus, it is preferable to transfect the host cells with a mixture of multiple different CTGs, preferably dCTGs. The number of different dCTGs is preferably five or more. Moreover, it is preferred that, among themselves, the multiple dCTGs show amino acid differences that

30   essentially extend throughout the polypeptide chains of the encoded sequences. The dCTGs would be selected to maximize amino acid differences and, at the

same time, make sure that differences are found all along the polypeptide chain. It would thus not be preferable to select a battery of deletions all from within the same domain of the polypeptide chain.

According to a protocol which utilizes 10^7 irradiated cells for immunization containing five separate dCTGs, five groups of 2×10^6 cells are included in one inoculate, each group of 2×10^6 having been transfected with a separate dCTG from the total set of five CTGs that are cognate to a particular proto-oncogene.

Selection of Non-Transforming Cognate Transgenes

Non-transforming cognate transgene variants are most advantageously derived via deletion of a sequence essential for transformation. Unlike point mutations which are potentially reversible due to back mutations, deletion mutations are irreversible. Furthermore, deletion mutations do not possess the inherent disadvantage attaching to point mutations, namely, even though the requirement for generation of an acceptable cognate transgene is for a qualitative difference with the wild type, i.e., non-transforming versus transforming, any given point mutation may be neutral or else quantitative in its effect, that is, the mutation may reduce but not totally eliminate transformability. Thus, according to a preferred embodiment of the invention, a deletion is created in a region of the cognate transgene which encodes an amino acid sequence required for transformation. Consonant with non-transformability, the smallest deletion possible so as to leave intact the bulk of the antigenicity of the transgene product is selected.

The engineering of a cognate transgene deletion mutant that satisfies these criteria is facilitated by reports of structure-function relationship in oncogene-encoded proteins. Such reports serve to identify regions of oncoproteins that are essential for transformation, as opposed to regions which are either neutral or serve merely to modulate transformability. Although such reports are usually based on *in vitro* transformation assays, and are therefore independent of immune effects, these studies can be exploited to aid in the

construction of non-transforming dCTGs for use in the practice of the present invention.

The deletion mutant is engineered to include at least a part of the region identified as critical for transformation. In those cases where essential amino acids have been identified, the deletion will span these residues. The engineering of any desired deletion can be readily accomplished by polymerase chain reaction (PCR) according to conventional PCR techniques, based upon the known nucleotide sequence of the unmutated cognate transgene.

The following describes a representative protocol for deriving a non-transforming dCTG of the smallest possible deletion, for use in the practice of the present invention. A test dCTG, engineered on the basis of known or ascertained transformation-specific domains, and driven by the strongest possible promoter, is used to transfect murine 3T3 cells. A sister culture of 3T3 cells is also transfected, with non-deleted CTG. Each CTG or dCTG cell culture is inoculated into nude mice, in the absence of any treatment to render the cells non-dividing. Those dCTGs which do not yield tumors in the mice even after prolonged observation are then utilized as transgenes for the biopsied human cells which, upon transfection with the transgene, will serve as a cellular vaccine according to the practice of the present invention. The dCTGs are selected with the smallest deletion mutant consonant with non-transformability.

Some CTGs representing xenogenic proto-oncogenes may not be tumorigenic in the 3T3/nude mouse assay. For any such non-transforming CTG, it is not essential to generate a dCTG. However, even given non-tumorigenicity in nude mice, it may be desirable to opt for generation of a deletion mutant when the transgene is based upon a xenogenic proto-oncogene. In such cases, the deletion would be engineered so as to remove the homologous region to that deleted in the particular dCTG that corresponds to the deletion in the corresponding retroviral oncogene dCTG.

Even though the transgene construct may comprise mutant oncogene or proto-oncogene DNA which is nontransforming, it is nevertheless preferable, as a safety measure, to treat the transfected cells to render them non-

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dividing before inoculation back into the host. The cells are irradiated with a
radiation dosage sufficient to render them non-dividing.

Oncogenicity Assay of Cognate Transgenes

As a further safety measure, the oncogenicity of a given dCTG
is preferably thoroughly tested prior to infection of the human host cells which
are used as cellular immunogens according to the practice of the present
invention. For example, an oncogenicity testing regimen may take the form of
three separate assays: (i) dCTG transfection of NIH 3T3 cells, followed by
inoculation into nude mice; (ii) dCTG transfection of human fibroblasts,
followed by inoculation into nude mice; and (iii) dCTG transfection of human
fibroblasts, followed by an *in vitro* test of anchorage-dependent growth. In
principle, all three should be negative to validate the use of any given dCTG in
the vaccination method of the present invention.

According to the oncogenicity assay (i), after stable transfection
of NIH 3T3 cells with the test dCTG, the transfectants are inoculated into nude
mice. Tumorigenicity of the transfectants in the mice is then evaluated
according to standard protocols.

According to oncogenicity assay (ii), human fibroblasts are
transfected with the test dCTG as proposed in the above human immunization
protocol. After stable dCTG transfection of human fibroblasts, however, rather
than carrying out X-irradiation of the transfectants to render them non-dividing,
followed by inoculation of the irradiated transfectants back into the human host,
the transfectants are directly inoculated into nude mice as a direct test of
tumorigenicity. Given the greater susceptibility of murine 3T3 cells to
oncogenic transformation, *vis a vis* primary human or murine transfectants
fibroblasts, assay (ii) is probably much less sensitive than assay (i), but does
have the advantage of offering a direct test of dCTG oncogenicity in human
cells.

According to oncogenicity assay (iii), non-irradiated dCTG-
transfected human fibroblasts are assayed for anchorage-dependent growth, *i.e.*

colony formation in soft agar, as a test of dCTG transforming potential in human cells. Anchorage independence, as defined by the ability of cells to grow when suspended in semisolid medium, is a common phenotype acquired by human tumor cells, particularly those tumor cells of mesenchymal origin,

5   such as fibrosarcomas. While assay (iii) has no *in vivo* readout, it offers an independent test of the critical issue of dCTG oncogenicity in human cells.

The oncogenicity assays are performed according to published protocols. Assay (i), comprising dCTG transfection of NIH 3T3 cells followed by inoculation into nude mice, may be performed according to the protocol of

10   Stevens *et al.*, *Proc. Natl. Acad. Sci. USA* (1988), 85:3875-3879, including DNA transfection by the calcium phosphate coprecipitation method of Manohaven *et al.*, *Carcinogenesis* (1985), 6:1295-1301. Accordingly, NIH 3T3 cells (7.5×10^5 cells per 100-mm dish) are exposed to a calcium phosphate-DNA coprecipitate (40 μg of genomic DNA plus 3 μg of pSV2neo per dish) for

15   4 hours. Two days later, each dish is trypsinized and reseeded into a 175-cm^2 flask. For the next 10 days, cultures are selected in G418 (400 μg/ml), and the flasks are then trypsinized and cells are replated in the same flask to disperse the G418-resistant colonies into a diffuse lawn of cells. Two days later, the cells are harvested and washed with serum-free medium prior to injection. One

20   injection of 5×10^6 cells into the right flank and one injection of 1×10^7 cells into the left flank, each in a volume of 200 μl, are done on each nude mouse. Injection sites are monitored at 3- or 4-day intervals for 100 days. The sites are scored for the number of tumors induced per injection site.

Oncogenicity assay (ii), whereby dCTG transfection of human

25   fibroblasts followed by inoculation into nude mice, is carried out in the same manner as assay (i) except that for assay (ii) the human fibroblast transfectants are substituted for the murine 3T3 transfectants.

Assay (iii), involves a test of the *in vitro* anchorage-dependent growth of dCTG-transfected human fibroblasts. The assay is carried out as

30   described in Stevens *et al.*, *J. Cancer Res. and Clin. Oncol.* 1989, 115:118-128. 1×10^5 cells are seeded per 60-mm dish into 0.33% Noble agar over a

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6-ml 0.5% agar base layer in Hams F10 supplemented with 6% fetal bovine serum. A portion of the agar suspension is diluted with Hams F10 plus 6% fetal calf serum to 200 cells/5 ml to determine the cloning efficiency of these cells when seeded into plastic 60-mm dishes. Agar dishes are fed with 1 ml

5   Hams F10 supplemented with 6% fetal bovine serum on the 1st and 15th day after seeding. Four weeks after seeding, all agar colonies > 75 μm in diameter are counted and the colony counts are normalized to the plating efficiencies which aliquots of the initially seeded cells showed on plastic. This comparison, or normalization, of the agar colony counts to the plastic dish colony counts is

10   useful in identifying and correcting for any mechanical artifacts which might result from the seeding into agar of dead cells that had persisted from the initial transfection treatment or from heat-induced cell death, which might have occurred while suspending cells in molten agar during the process of seeding the agar dishes.

15   The following is a partial list of various deletions which, based upon published accounts of experiments with human or animal cells, are believed to render the identified CTG non-tumorigenic.

Table 2

Deletion Mutations Rendering Indicated Gene Non-Transforming

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
Akt-2 (c-akt) (mouse)	M95936; SEQ ID NO:3 (*Mus musculus* serine/threon ine kinase)	480	148-234	Bellacosa *et al.*, *Science* (1991), 254:274-278; Bellacosa *et al.*, *Oncogene* (1993), 8(3):745-54.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-*neu* (c-erbB-2) (rat)	M11730; SEQ ID NO:4 (human tyrosine kinase-type receptor (HER2) gene	1255	1-731	Bargmann *et al.*, *EMBO* (1988), 7(7):2043-52; Bernards *et al.*, *Proc. Natl. Acad. Sci. USA* (1987), 84(19):6854-8.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
mdm-2 (human)	U33199; SEQ ID NO:5 (human mdm2-A mRNA); U33200; SEQ ID NO:6 (human mdm2-B mRNA); U33201; SEQ ID NO:7 (human mdm2-C mRNA); U33202; SEQ ID NO:8 (human mdm2-D mRNA); U33203;	489	9-155	Dubs-Poterszman, *Oncogene* (1995), 11(11):2445-50.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-*myb* (human)	J02012; SEQ ID NO:10 (proviral oncogene v-*myb*)	640	275-327	Kalkbrenner *et al.*, *Oncogene* (1990), 5(5):657-61.
c-*myc* (human)	X00364; SEQ ID NO:11 (human c-*myc* oncogene)	439	129-144	Sarid *et al.*, *Proc. Natl. Acad. Sci. USA* (1987), 84(1):170-3.
v-*ras* (Harvey Murine Sarcoma Virus)	M77193; SEQ ID NO:12 (Rat sarcoma virus v-*ras* oncogene)	189	32-44	Zhang *et al.*, *Science* (1990), 249:162-5 (1990)

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CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
v-src (Rous Sarcoma Virus)	U41728; SEQ ID NO:13 (RSV Schmidt-Ruppin A clone SRA-V; v-src gene)	526	430-433	Bryant et al., Mol. Cell. Bio. (1984), 4(5):862-6.
c-yes (chicken)	D00333; SEQ ID NO:14 (human c-yes-2 gene)	541	438-441	Zheng et al.; Oncogene (1989), 4(1):99-104.

Engineering of Vectors for Host Cell Transfection

The engineering of vectors for expression of a particular CTG. preferably a dCTG, is based on standard methods of recombinant DNA technology, i.e. insertion of the dCTG via the polylinker of standard or

5   commercially available expression vectors. The dCTG is operably linked to a strong promoter. Generally speaking, a "strong" promoter is a promoter which achieves constitutively high expression of the dCTG in the transfected cells. Each promoter should include all of the signals necessary for initiating transcription of the relevant downstream sequence. These conditions are

10   fulfilled, for example, by the pBK-CMV expression vector available from Stratagene Cloning Systems, La Jolla, CA (catalog no. 212209). The pBK-

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CMV vector contains the cytomegalovirus (CMV) immediate early promoter. dCTGs xenogenic with respect to a particular target proto-oncogene may be isolated by conventional nucleic acid probing techniques, given the availability of a highly homologous probe represented by the cognate retroviral oncogene

5   and/or the human proto-oncogene itself.

Collection of Host Cells for Transfection

The host cells which may be transfected to derive the cellular immunogens of the present invention must express class I MHC and be susceptible to isolation and culture. Fibroblasts express class I MHC and may

10   be cultured. Accordingly, punch biopsies of host human skin are performed to harvest fibroblasts. Punch biopsies can be performed by a competent physician as a standard clinical procedure. Each biopsy yields a starting population of 1-2 $X\ 10^7$ cells that would proliferate in culture. Methods for the preparation of tissue cultures of human fibroblasts are well developed and widely used. *See*,

15   Cristofalo and Carpenter, *J. Tissue Culture Methods* (1980), 6:117-121, the entire disclosure of which is incorporated herein by reference. Essentially, skin obtained by punch biopsy is washed using an appropriate wash medium, finely minced and cultured in a suitable culture medium, such as Dulbecco's Modified Eagle Medium (DMEM), under CO_2 at 37°C. The cells are trypsinized with

20   a trypsin solution and transferred to a larger vessel and incubated at 37°C in culture fluid.

Host Cell Transfection

The expression vector carrying the dCTG is used to transfect biopsied host cells according to conventional transfection methods. One method

25   of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. *See* McCutchin and Pagano, *J. Natl. Cancer Inst.* (1968) 41:351-7. Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca^{++} to a phosphate-containing DNA solution. The resulting precipitate

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apparently includes DNA in association with calcium phosphate crystals. These crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descen-

5   dants. *See* Graham *et al.*, *Virology* (1973), 52:456-467 and *Virology* (1974), 54:536-539.

Preferably, transfection is carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)

10   or related liposome-forming materials. *See* Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7417 (DNA-transfection); Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989), 86:6077-6081) (RNA-transfection). One preferred technique utilizes the LipofectAMINE™ Reagent (Cat. No. 18324-012, Life Technologies, Inc., Gaithersburg, MD) which is a 3:1 (w/w) liposome

15   formulation   of   the   polycationic   lipid   2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate), and the neutral lipid

20   dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. Transfection utilizing the LipofectAMINE™ Reagent is carried out according to the manufacturer's published protocol. The protocol (for Cat. No. 18324-012) provides for either transient or stable transfection, as desired.

The advantage of transient expression is its rapidity, *i.e.* there is

25   no requirement for cellular proliferation to select for stable integration events. This rapidity could conceivably be of major clinical importance, in cases of an already metastatic tumor burden, wherein the weeks required for selection of stable transfectants may simply not be available to the clinician.

There are, nonetheless, two general disadvantages to the use of

30   transient transfection. The first is that expression usually peters out after a few days, in contrast to the continual expression in the case of stable transfection.

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This is not particularly crippling in terms of our immunization protocol. The inoculated, irradiated cells used for immunization would likely not survive *in vivo* for more than 4 or 5 days, in any case. Thus the nominal advantage accruing to stable transfection, that of a long-duration expression by the progeny

5   of the parental inoculated cell, is not of particular relevance in the case of the immunizing regime described herein, which is based on the use of non-dividing, probably short-lived cells.

A second disadvantage of transient transfection resides in the fact that it yields a cell population, only a subset of which has actually been

10 transfected and thus expresses the protein encoded by the transgene. This problem is obviated in the case of stable transfection, wherein over time one can develop a pure population of transfectants via selection for a resistance marker, such as *neo*, under conditions of clonal proliferation of the initial stable transfectants, *i.e.* daughter cells of transiently transfected cells lack the

15 transgene, in contrast to the case with stable transfectants. In the situation where there is sufficient time to effect immunization based on stably transfected cells, the progeny of all transfected clones would be utilized, not just the progeny of a single clone, as is sometimes done for detailed biochemical and molecular analyses of gene expression. Clearly the more clones utilized, the

20 more quickly one can arrive at the requisite number of cells to be used for immunization.

Percentage of Cells Exhibiting dCTG Expression

The percentage of cells exhibiting dCTG expression may be determined by an immunohistology assay. In this procedure, a small number

25 of cells (~ 500) from the harvested pellet following centrifugation of transfected cells are deposited on a cover slip and fixed with cold acetone. At this point, a standard immunohistological assay is carried out with the cells on the cover slip, *i.e.* addition of a primary monoclonal antibody reactive to the dCTG-encoded protein, followed by the addition of a developing antibody, *e.g.* a

30 fluorescent tagged antibody reactive to the primary monoclonal antibody.

Measurement of the percentage of cells scoring as dCTG-positive in the fluorescent assay allows a determination of the number of positive transfectants in the starting culture, and thus the number of total cells to be used for immunization to arrive at the desired number of dCTG-positive cells to be
5   inoculated in the patient.

If, as would be almost certain, the percentage of cells scoring as dCTG-positive is less than one hundred percent, one can simply increase the number of cells to be used for immunization, so as to include the desired number of transfectants. The non-transfected cells in the immunizing population
10   would simply represent x-irradiated, autologous fibroblasts that would constitute no danger to the patient.

Transfectant Irradiation

Prior to return to the host, the transfected cells are preferably irradiated. The transfectants are irradiated with a radiation dose sufficient to
15   render them non-dividing, such as a dose of 25 By or 2500R. The cells are then counted by trypan blue exclusion, and about 2×10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

Vaccination Procedure

20   The transfected cells are returned to the host to achieve vaccination. The cells may be reimplanted at the same body site from which they were originally harvested, or may be restored to a different site.

It is the object of the present invention to generate a systemic tumor immune response, so as to fight metastasis formation wherever any
25   metastases are found. Accordingly, there is no reason to inject the transfected cells at the same body site from which they were taken. Intramuscular or subcutaneous inoculation at a distal site would suffice to yield a systemic response. Thus, patients are preferably vaccinated by subcutaneous inoculation of the transfected cells.

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For s-*crc* overexpression associated with colon carcinoma, partial venous inoculation is preferred, as the liver is a frequent site of metastases. For vaccinating against breast cancers and lymphomas, systemic immunization is preferred.

As a general rule, it is desirable to generate the strongest immune response consistent with clinical monitoring of no adverse side effects, *i.e.* multiple rounds of inoculation with, for example 10^7 cells, at each round. The number of rounds of inoculation is selected accordingly. The efficacy of the inoculation schedule may be monitored by a delayed hypersensitivity reaction administered to the patient. A course of about up to 10 inoculations, at 2-3 week intervals, may be utilized. It may be appreciated that the inoculation schedule may be modified in view of the immunologic response of the individual patient, as determined with resort to the delayed-type hypersensitivity (DTH) reaction.

Patient Response Monitoring by Delayed-type Hypersensitivity Reaction

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction. DTH has been used clinically (Chang *et al.* (1993), *Cancer Research* 53:1043-1050). To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 cells in a volume of 0.1 ml Hanks buffered saline solution (HBSS) are inoculated intradermally into the host. Induration is measured 48 hours later, as an average of two perpendicular diameters (responses of greater than ≥ 2 mm is considered positive).

One advantage to the DTH assay is that it can independently assess the induction of T cell reactivity to (i) the transfectants used for immunization (*i.e.* the set of 5 or more dCTGs chosen for immunization purposes, each containing non-self determinants) and (ii) transfectants, as transfected with the human dCTG itself containing only self determinants. Thus, the induction of reactivity to the transfectants used for immunization establishes that the immunizing transfectants are in fact immunogenic, that is, the patient has not exhibiting a much weakened capacity for immune response.

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If the patient is demonstrably capable of response to the immunizing transfectants, then skin testing with the dCTG (human) transfectants would establish whether or not reactivity to the human proto-oncogene encoded product had been induced. According to the practice of the invention, inoculation of the

5   immunizing transfectants would continue for at least as long as the induction of reactivity to the human proto-oncogene-encoded protein occurs.

The practice of the invention is illustrated by the following nonlimiting examples.

Example 1

10   ## Immunization of Chickens Against c-src(527)-Induced
Tumors By Vaccination with v-src DNA

A.   Genes

The oncogene c-src(527) is an activated form of chicken c-src. Its protein product pp60$^{c\text{-}src(527)}$ differs from the protein product of c-src, pp60$^{c\text{-}}$

15   ^{src}, by only a single amino acid substitution, phenylalanine for tyrosine at residue 527 (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73). This substitution eliminates the negative regulatory influence exerted on pp60$^{c\text{-}src}$ phosphokinase activity by the enzymatic phosphorylation of the position 527 tyrosine. The protein product of v-src, pp60$^{v\text{-}src}$, shows a number of sequence differences with

20   pp60$^{c\text{-}src}$ (Takeya and Hanafusa, (1983) *Cell* 32, 881-890), including scattered single amino acid substitutions within the first 514 residues and a novel C terminus of 12 amino acids (residues 515-526), in place of the nineteen C terminal amino acids of pp60$^{c\text{-}src}$ (residues 515-533). Both the v-src-positive plasmid, pMvsrc, and the c-src(527)-positive plasmid, pcsrc527, were originally

25   shown (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73) to transform murine NIH 3T3 cells in culture. However, the v-src-induced transformants exhibited a more rapid or more extensive colony growth in soft agarose than the c-src(527)-induced transformants, as well as a usually shorter latency of tumor formation in nude mice (*id.*).

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B.   Plasmids

1.   pvSRC-C1

The pVSRC-C1 plasmid was prepared as described by Halpern *et al.*, (1991) *Virology* 180, 857-86. Essentially, the plasmid was derived from the pRL^v-*src* plasmid (Halpern *et al.*, (1990) *Virology* 175, 328-331) by subcloning the v-*src*(+) *XhoI-EcoRI* fragment of the latter into the multiple cloning sequence of pSP65 (Melton *et al.*, (1984) *Nucleic Acids Res.* 12, 7035-7056) which had been cleaved with *SalI* and *EcoRI*; since ligation of the *XhoI* overhang at the *SalI* site destroys both recognition sequences, subsequent removal of the v-*src*(+) insert from the vector was achieved by digestion with *EcoRI* and with *HindIII*, which cleaves at a position in the multiple cloning sequence adjacent to the *SalI* site. The pVSRC-C1 plasmid was restricted with *EcoRI* and *HindIII*, so as to liberate the tumorigenic insert. This insert included the v-*src* oncogene of the subgroup A strain of Prague RSV, as flanked downstream by a portion of the long terminal repeat (LTR) of RSV (from the 5' start of the LTR, to the single *EcoRI* site).

2.   pMv*src*

The pMv*src* plasmid was generously provided by Dr. David Shalloway, Cornell University, Ithaca, NY. The plasmid is prepared according to Johnson *et al.*, (1985) *Mol. Cell. Biol.* 5, 1073-1083. Briefly, the 3.1-kb *BamHI-BgII* Schmidt Ruppin A v-*src* fragment from plasmid pN4 (Iba *et al.*, (1984) *Proc. Nat. Acad. Sci. USA* 81, 4424-4428) is inserted into the pEVX plasmid (Kriegler *et al.*, (1984) *Cell* 38,483-491) at a *BgII* site lying between two Moloney murine leukemia virus (MoMLV) long terminal repeats (LTRs). This fragment contains 276 bp of pBR322 DNA from the pBR322 *BamHI* to *SalI* sites followed by 2.8 kb of Rous sarcoma virus (RSV) DNA from the *SalI* site that is about 750 bp upstream of the *env* termination codon down to the *NruI* site that is about 90 bp downstream of the v-*src* termination codon. (The

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*Nru*I site is converted to a *Bg*/II site in the construction of pN4.) Ligation is performed by using a 10:1 insert-vector DNA fragment molar ratio.

The pMv*src* plasmid was restricted with NheI, so as to liberate a tumorigenic fragment. The fragment included the v-*src* oncogene of the

5   subgroup A strain of Schmidt-Ruppin RSV, as flanked upstream by most of the Moloney murine leukemia virus (MoMLV) LTR (from the *Nhe*I site near the 5' start of the LTR, to the 3' end of this LTR) and downstream by a small portion of the MoMLV LTR (from the 5' start to the *Nhe*I site).

3.   pc*src*527

10   The pc*src*527 plasmid is prepared according to Kmiecik and Shalloway, (1987) *Cell* 49, 65-73. Briefly, a plasmid is constructed by cleaving expression vector pEVX (Kriegler *et al.*, (1984) *Cell* 38,483-491 at its unique *Bg*III site lying between two MoMLV LTRs and inserting the 3.2 kilobase (kb) pair *Bam*HI-*Bg*III hybrid *src* fragment from plasmid pHB5 in the proper

15   orientation. This fragment contains sequences from pBR322, the SRA *env* 3' region, SRA v-*src*, *src* from recovered ASV, and chicken c-*src*. The *Bg*III site is generated by insertion of a linker at the *Sac*I site about 20 bp downstream from the c-*src* termination codon. The restriction map of pMHB5 contains the MoMLV splice donor about 60 bp downstream from the 3'end of the upstream

20   LTR and the v-*src* splice acceptor about 75 bp upstream from the *src* ATG.

Plasmid pMHB5527 is constructed by inserting the synthetic double-stranded DNA oligomer

```
5'        CCAGTTCCAGCCTGGAGAGAACCTATA (SEQ ID NO:1)        3'
3'    TCGGGGTCAAGGTCGGACCTCTCTTGGATATCTAG (SEQ ID NO:2)    5'
```

25   into pMHB5 between the *Ban*II site at c-*src* codon 524 and the downstream unique *Bg*III site. This alters the TAC Tyr 527 codon to a TTC Phe codon while preserving the remaining c-*src* coding region. Equimolar amounts of the double-stranded oligomer and three gel-purified tandem restriction fragments from pMHB5 are ligated in one reaction, which contains the following: the

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oligomer with *Ban*II and *Bg*III complementary ends, the 3 kb *Bg*III-*Bg*II (*Bg*II in the pEVX ampicillin resistance gene) partial digest fragment, the adjacent 6.1 kb *Bg*II-*Bg*II (downstream Bg*II* in c-*src*) fragment, and the 0.38 kb *Bg*II-*Ban*II (*Ban*II at c-*src* codon 524) fragment.

5   Plasmid pc*src*527 is constructed by replacing the 2 kb *Sal*I (in *env*)-*Mlu*I (in c-*src*) fragment in plasmid pMHB5527, with the homologous fragment from plasmid p5H. This fragment contains the coding sequence for the c-*src* amino region (codons 1 to 257) that have been isolated by molecular cloning of a c-*src* provirus and previously shown by sequencing to contain

10   authentic c-*src* sequence without the mutation at codon 63 (Levy *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83, 4228-4232). Equimolar amounts of complementary gel-purified *Sal*I-*Mlu*I fragments from p5H and the other plasmids are ligated.

          The pc*src*527 plasmid was restricted with *Nhe*I, so as to liberate

15   a tumorigenic fragment. The tumorigenic fragment included the c-*src*(527) oncogene, as flanked by the same LTR complement as in pMv*src*.

C.   <u>Animals</u>

          Chickens of two closed lines, SC and TK, were utilized. These lines differ at the major histocompatibility (*B*) complex (B^2/B^2 for the SC line,

20   B^{15}/B^{21} for the TK line). Embryonated eggs were obtained from Hyline International (Dallas Center, IA). All chickens were hatched at the University of New Hampshire Poultry Research Farm and housed in isolation.

D.   <u>Tumor Induction by Plasmid DNA</u>

          Tumors were induced by subcutaneous inoculation in the wing

25   web of a *src*-positive plasmid according to the technique described by Fung *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80, 353-357 and Halpern *et al.*, (1990) *Virology* 175, 328-331. Of the three tumorigenic plasmids utilized here, all were adjusted, prior to inoculation, to a concentration of 100 μg of enzyme--restricted DNA per 100 μl of phosphate-buffered saline. The conditions of

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inoculation used for particular experiments (age of chicken at time of inoculation, amount of plasmid, etc.) are indicated below.

E.   Growth of Primary (wing web) Tumors in TK or SC Chickens
            Inoculated with pVSRC-C1, pMv*src* or pc*src*527

5   Individual 1-day-old chickens of line TK or of line SC were inoculated with 100 μg of either pVSRC-C1, pMv*src* or pc*src*527. The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated with an individual *src*-positive construct was computed as the sum of the diameters of the primary tumors divided by the

10   number of chickens surviving to that point. The results are shown in Fig. 1A (line TK) and Fig. 1B (line SC). The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for pc*src*527, italics for pVSRC-C1, bold typeface for pMV*src*). Error bars (unless obscured by the

15   symbol) indicate standard error.

F.   Growth of Challenge (wing web) Tumors in Test and Control
            Line TK Chickens Under Conditions of Priming and Homologous
            Challenge with pc*src*527, or Priming and Homologous Challenge
            with pVSRC-C1

20   Growth of challenge (wing web) tumors in test and control line TK chickens was determined under conditions of (i) priming and homologous challenge with pc*src*527, or (ii) priming and homologous challenge with pVSRC-C1. Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch

25   with 200 μg of construct. The mean challenge tumor diameter was computed as described in the preceding section. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming and homologous challenge with pc*src*527 (Fig. 2, panel A) and priming and homologous challenge with pVSRC-C1 (Fig. 2, panel B)

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(standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *(p<0.05), **(p<0.01), ***(p<0.001). The statistical

5   comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where p<0.05. Error bars indicate standard error.

G.   Growth of Challenge (wing web) Tumors in Test and Control
10   line TK chickens under Conditions of Priming with pVSRC-C1
                and Heterologous Challenge with pcsrc527, or Priming with
                pcsrc527 and Heterologous Challenge with pVSRC-C1

Growth of challenge (wing web) tumors in test and control line TK chickens, was determined under conditions of (i) priming with pVSRC-C1

15   and heterologous challenge with pcsrc527, or (ii) priming with pcsrc527 and heterologous challenge with pVSRC-C1. Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as described in Section E. At each time point the ratio

20   of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming with pVSRC-C1 and heterologous challenge with pcsrc527 (Fig. 3, panel A) and priming with pcsrc527 and heterologous challenge with pVSRC-C1 (Fig. 3, panel B) (standard typeface for control group, bold typeface for test group). Statistical comparisons were made

25   between test and control groups at a particular time point as described in the preceding section [*(p<0.05), **(p<0.01), ***(p<0.001), for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, p<0.05. Error bars indicate standard error.

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H.   <u>Discussion</u>

In a direct comparison of the growth of tumors induced in line
TK by either pMvsrc or pVSRC-C1, a similar pattern of relatively rapid
regression was observed. This result established that the difference in LTR
5   complement between these two v-src positive constructs did not exert a major
influence on the tumor growth pattern in the TK line (Fig. 1, panel A). By
contrast, much more extensive and persistent tumor growth resulted from
inoculation of TK chickens with the pcsrc527 construct (Fig. 1, panel A). The
relatively greater growth capacity of tumors induced by this construct indicated
10   that in the TK line, the c-src(527) oncogene is much more highly tumorigenic
than the v-src oncogene. This difference did not, however, generalize to the SC
line (Fig. 1, panel B). The SC line was chosen for comparison with the TK
line on the basis of earlier observations (Halpern *et al.*, (1993) *Virology* 197,
480-484) that v-src DNA-induced tumors engender a much weaker tumor
15   immune response in line SC than in line TK. Whereas the growth of pcsrc527-
induced primary tumors was virtually indistinguishable in the two lines, the
growth of the v-src-induced tumors was considerably greater in the SC than in
the TK line (Fig. 1). Thus v-src, but not c-src(527), gives rise to primary
tumors whose growth patterns differ in the two lines analyzed here.

20   Only minimal protection against homologous challenge was
observed under conditions of priming to c-src(527) DNA, indicative of the
induction of a relatively weak tumor immune response (Fig. 2, panel A; a
statistically significant lowering of challenge tumor growth in the test versus the
control chickens was observed at only one time point). By contrast, the v-src
25   DNA-primed chickens showed excellent protection against the homologous
tumor challenge (Fig. 2, panel B).

Priming with v-src DNA engenders a relatively greater degree of
protection against challenge with c-src(527) DNA, than that afforded by priming
with c-src(527) DNA itself (Fig. 3, panel A). The degree of protection was
30   weaker than that determined (Fig. 2, panel B) for the case of priming and
homologous challenge with v-src DNA. Only marginal protection was

- 50 -

observed, however, when the heterologous challenge protocol was carried out in the reverse order (Fig. 3, panel B). These results demonstrate that induction of reactivity to an antigenicity specified in tumor cells by an overexpressed proto-oncogene can confers tumor immunity.

5

Example 2

Vaccination Protocol

The following is a representative vaccination protocol according to the present invention.

A.   Skin Punch Biopsy

10   A punch biopsy of skin is obtained by a trained physician following standard medical practice.

B.   Preparation of Primary Fibroblast Culture

Under sterile conditions, the skin obtained by punch biopsy is put in a tube with 10 ml of the following wash medium: Dulbecco's Modified

15 Eagle Medium (DMEM), containing sodium bicarbonate (30 ml/liter of a 5.6% solution) and penicillin/streptomycin (2 ml/liter of a pen-strep stock solution containing 5000 units penicillin and 5000 μg of streptomycin/ml, pH 7.2-7.4.). In a sterile hood, the skin biopsy is added to a Petri dish, and then transferred several times to new Petri dishes containing the same wash medium. The

20 biopsy is then finely minced with two scalpels, and 2-4 pieces (<1 mm^3) of the minced biopsied are placed in the middle part of one or more T25 flasks. The flask is placed in a tissue culture incubator at 37°C for one half hour with the cap firmly closed, then opened for 10 minutes. The following culture medium is prepared: DMEM containing sodium bicarbonate; antibiotics; and 10% fetal

25 calf serum containing 2.5 μg/ml fungizone, 40 μg/ml gentamicin, and 1% glutamine(3% W/V). Two ml of the culture medium is then added to the flask, and the flask is incubated at 37°C (5% CO_2), with the cap lightly unscrewed. The flask is left for three days without moving so as to obtain adhesion of the

separate pieces of skin to the plastic. Afterwards, the medium is changed two times per week over a 3-4 week period always adding 2-3 ml of medium. To trypsinize the skin cell culture, one needs zones of confluence. After aspirating the culture medium, 5 ml of the Puck's Saline A/EDTA solution (0.4 g EDTA to 1 liter of Puck's Solution A) is added and immediately aspirated. Then 1 ml of trypsin solution (0.05/0.02% trypsin in PBS, without Ca++ or Mg++) is added and incubated for 5 min at 37°C, at which time 2 ml of culture fluid is added to stop the action of the trypsin. The cells are then transferred to a larger flask (T75) and incubated at 37°C in 15 ml of culture fluid, which is changed every 2 days.

C.   Fibroblast Transfection

The fibroblasts (2 X 10^5 cells) are washed twice in DMEM without serum or antibiotics. A LipofectAMINE™-DNA solution is prepared by mixing in tube #1 mix 400µl DMEM and 10µl of dCTG vector DNA (1µg/ul). In tube #2, 400 µl DMEM and 25 Ml of LipofectAMINE Reagent (Life Technologies, cat. no. 18324-012) are mixed. The contents of tube #1 and #2 are mixed together and are then left sitting at room temperature for 30 hours. Then, 3.2 ml of the LipofectAMINE™-DNA solution is added to the cells. The cells are incubated for six hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with growth medium and incubated for an additional 24 hours at 37°C

D.   Transfectant Irradiation

Transfectants are irradiated to a dose of 25 By or 2500R. the cells are then counted by trypan blue exclusion. 2 X 10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

E.   Vaccination

Patients are vaccinated by subcutaneous inoculation of 2 X 10^7 irradiated cells at 2-3 week intervals. A shorter or longer regimen is used,

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depending upon the results of delayed type hypersensitivity (DTH) reaction monitoring (described below).

F.   Patient Assessment by DTH Monitoring

Patients are assessed for reactivity to the irradiated transfectants

5   by a test of skin reactivity in a DTH reaction, as described by Chang *et al.*
(1993), *Cancer Research* 53:1043-1050.   To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 transfected irradiated cells in a volume of 0.1 ml HBSS are inoculated intradermally. Induration is measured 48 hours later, as an average of two perpendicular diameters. Responses of

10   greater than 2 mm are considered positive.

Example 3
v-*myc* Transfection of Murine Fibroblasts

A.   Vector Preparation

The v-*myc* retroviral oncogene of avian myelocytomatosis virus

15   MC29 (Land *et al.* (1983), *Nature* 304:596-602) was obtained from the American Type Culture Collection, Rockville, MD, 20852, as the pSVv-*myc* vector (ATCC No. 45014). The v-*myc*-positive *Eco*RI-*Kpn*I fragment of pSVv-*myc* was ligated into the polylinker sites of the pBK-CMV plasmid (Stratagene Cloning Systems, La Jolla, CA).

20   B.   Cell Transfection

Stable transfection using the pBK-CMV-v-*myc* vector was carried out on a line of A31 fibroblasts (Balb/c origin), obtained from the ATCC. 2 X 10^5 cells were seeded in a 100 mm/dish and allowed to grow for 18-20 h (RPMI 1640 medium and 10% fetal bovine serum), at which time the cells

25   reached 50-70% confluence. The cells were then washed twice in Dulbecco's Modified Eagles Medium (without serum or antibiotics). A LipofectAMINE™-DNA solution was prepared according to Example 2.C., with the pBK-CMV-v-

myc vector DNA, and 3.2 ml of the LipofectAMINE™-DNA solution added to
the cells. The cells were then incubated for 6 hours at 37°C, washed once with
Hank's Balanced Salt Solution, and then refed with the growth medium and
incubated for an additional 24 hour at 37°C. Thereafter, the cells were fed
5   once every two days with growth medium containing 250 μg/ml geneticin
(G418; Gibco BRL cat. no. 11811) as the selective marker. Within two weeks,
colonies were picked and expanded into permanent cell lines. The cells were
then washed and collected by centrifugation.

It should be noted that the procedure for transient transfection is
10   the same, through the point of incubation with the Lipofectamine™-DNA
solution. Thereafter, the cells are washed and incubated for 72 hours in growth
medium.

All references cited with respect to synthetic, preparative and analytical
procedures are incorporated herein by reference.
15   The present invention may be embodied in other specific forms without
departing from the spirit or essential attributes thereof and, accordingly,
reference should be made to the appended claims, rather than to the foregoing
specification, as indication the scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Allegheny University of the Health Sciences
                  Halpern, Michael S.
                  England, James M.

    (ii) TITLE OF INVENTION: CANCER VACCINE

    (iii) NUMBER OF SEQUENCES: 14

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Seidel, Gonda, Lavorgna & Monaco, P.C.
        (B) STREET: Suite 1800, Two Penn Center Plaza
        (C) CITY: Philadelphia
        (D) STATE: PA
        (E) COUNTRY: USA
        (F) ZIP: 19102

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 60/010,262
        (B) FILING DATE: 19-JAN-1996

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Monaco, Daniel A.
        (B) REGISTRATION NUMBER: 30,480
        (C) REFERENCE/DOCKET NUMBER: 7933-33 PC

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (215) 568-8383
        (B) TELEFAX: (215) 568-5549

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCAGTTCCAG CCTGGAGAGA ACCTATA   27

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GATCTATAGG TTCTCTCCAG GCTGGAACTG GGGCT   35

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1599 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GAGACTGTGC CCTGTCCACG GTGCCTCCTG CATGTCCTGC TGCCCTGAGC TGTCCCGAGC      60

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CTCCACAAGC GTGGTGAATA CATCAAGACC TGGAGGCCAC GGTACTTCCT GCTGAAGAGC      180

GACGGCTCCT TCATTGGGTA CAAGGAGAGG CCCGAGGCCC CTGATCAGAC TCTACCCCCC      240

TTAAACAACT TCTCCGTAGC AGAATGCCAG CTGATGAAGA CCGAGAGGCC GCGACCCAAC      300

ACCTTTGTCA TACGCTGCCT GCAGTGGACC ACAGTCATCG AGAGGACCTT CCACGTGGAT      360

TCTCCAGACG AGAGGGAGGA GTGGATGCGG GCCATCCAGA TGGTCGCCAA CAGCCTCAAG      420

CAGCGGGCCC CAGGCGAGGA CCCCATGGAC TACAAGTGTG GCTCCCCCAG TGACTCCTCC      480

ACGACTGAGG AGATGGAAGT GGCGGTCAGC AAGGCACGGG CTAAAGTGAC CATGAATGAC      540

TTCGACTATC TCAAACTCCT TGGCAAGGGA ACCTTTGGCA AAGTCATCCT GGTGCGGGAG      600

AAGGCCACTG GCCGCTACTA CGCCATGAAG ATCCTGCGAA AGGAAGTCAT CATTGCCAAG      660

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GGGACCCCGG AGTACCTGGC GCCTGAGGTG CTGGAGGACA ATGACTATGG CCGGGCCGTG     1080

GACTGGTGGG GGCTGGGTGT GGTCATGTAC GAGATGATGT GCGGCCGCCT GCCCTTCTAC     1140

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ACGCTCAGCC CCGAGGCCAA GTCCCTGCTT GCTGGGCTGC TTAAGAAGGA CCCCAAGCAG     1260

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TCCGAGGTCG ACACAAGGTA CTTCGATGAT GAATTTACCG CCCAGTCCAT CACAATCACA     1440

CCCCCTGACC GCTATGACAG CCTGGGCTTA CTGGAGCTGG ACCAGCGGAC CCACTTCCCC     1500
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CAGTTCTCCT ACTCGGCCAG CATCCGCGAG TGAGCAGTCT GCCCACGCAG AGGACGCACG   1560

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(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 4530 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AATTCTCGAG CTCGTCGACC GGTCGACGAG CTCGAGGGTC GACGAGCTCG AGGGCGCGCG   60

CCCGGCCCCC ACCCCTCGCA GCACCCCGCG CCCCGCGCCC TCCCAGCCGG GTCCAGCCGG   120

AGCCATGGGG CCGGAGCCGC AGTGAGCACC ATGGAGCTGG CGGCCTTGTG CCGCTGGGGG   180

CTCCTCCTCG CCCTCTTGCC CCCCGGAGCC GCGAGCACCC AAGTGTGCAC CGGCACAGAC   240

ATGAAGCTGC GGCTCCCTGC CAGTCCCGAG ACCCACCTGG ACATGCTCCG CCACCTCTAC   300

CAGGGCTGCC AGGTGGTGCA GGGAAACCTG GAACTCACCT ACCTGCCCAC CAATGCCAGC   360

CTGTCCTTCC TGCAGGATAT CCAGGAGGTG CAGGGCTACG TGCTCATCGC TCACAACCAA   420

GTGAGGCAGG TCCCACTGCA GAGGCTGCGG ATTGTGCGAG GCACCCAGCT CTTTGAGGAC   480

AACTATGCCC TGGCCGTGCT AGACAATGGA GACCCGCTGA ACAATACCAC CCCTGTCACA   540

GGGGCCTCCC CAGGAGGCCT GCGGGAGCTG CAGCTTCGAA GCCTCACAGA GATCTTGAAA   600

GGAGGGGTCT TGATCCAGCG GAACCCCCAG CTCTGCTACC AGGACACGAT TTTGTGGAAG   660

GACATCTTCC ACAAGAACAA CCAGCTGGCT CTCACACTGA TAGACACCAA CCGCTCTCGG   720

GCCTGCCACC CCTGTTCTCC GATGTGTAAG GGCTCCCGCT GCTGGGGAGA GAGTTCTGAG   780

GATTGTCAGA GCCTGACGCG CACTGTCTGT GCCGGTGGCT GTGCCCGCTG CAAGGGGCCA   840

CTGCCCACTG ACTGCTGCCA TGAGCAGTGT GCTGCCGGCT GCACGGGCCC CAAGCACTCT   900

GACTGCCTGG CCTGCCTCCA CTTCAACCAC AGTGGCATCT GTGAGCTGCA CTGCCCAGCC   960

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CGAGAGGTGA GGGCAGTTAC CAGTGCCAAT ATCCAGGAGT TTGCTGGCTG CAAGAAGATC   1260

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CCGCTCCAGC CAGAGCAGCT CCAAGTGTTT GAGACTCTGG AAGAGATCAC AGGTTACCTA   1380

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ATCCGGGGAC GAATTCTGCA CAATGGCGCC TACTCGCTGA CCCTGCAAGG GCTGGGCATC   1500

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AGCTGGCTGG GGCTGCGCTC ACTGAGGGAA CTGGGCAGTG GACTGGCCCT CATCCACCAT    1560

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TGCCACCAGC TGTGCGCCCG AGGGCACTGC TGGGGTCCAG GGCCCACCCA GTGTGTCAAC    1740

TGCAGCCAGT TCCTTCGGGG CCAGGAGTGC GTGGAGGAAT GCCGAGTACT GCAGGGGCTC    1800

CCCAGGGAGT ATGTGAATGC CAGGCACTGT TTGCCGTGCC ACCCTGAGTG TCAGCCCCAG    1860

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GGAATGGGGG CAGCCAAGGG GCTGCAAAGC CTCCCCACAC ATGACCCCAG CCCTCTACAG    3480

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TCGCCCCGAG AGGGCCCTCT GCCTGCTGCC CGACCTGCTG GTGCCACTCT GGAAAGGGCC    3660

AAGACTCTCT CCCCAGGGAA GAATGGGGTC GTCAAAGACG TTTTTGCCTT TGGGGGTGCC    3720

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CCTGCCTTCA GCCCAGCCTT CGACAACCTC TATTACTGGG ACCAGGACCC ACCAGAGCGG    3840

GGGGCTCCAC CCAGCACCTT CAAAGGGACA CCTACGGCAG AGAACCCAGA GTACCTGGGT    3900

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CAGGGGAACC TGCCATGCCA GGAACCTGTC CTAAGGAACC TTCCTTCCTG CTTGAGTTCC    4080

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CAGGGGAGAA TGGGTGTTGT ATGGGGAGGC AAGTGTGGGG GGTCCTTCTC CACACCCACT    4500

TTGTCCATTT GCAAATATAT TTTGGAAAAC                                     4530
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(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 891 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA     60

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TTGGATCAGG ATTCAGTTTC AGATCAGTTT AGTGTAGAAT TTGAAGTTGA ATCTCTCGAC    180

TCAGAAGATT ATAGCCTTAG TGAAGAAGGA CAAGAACTCT CAGATGAAGA TGATGAGGTA    240

TATCAAGTTA CTGTGTATCA GGCAGGGGAG AGTGATACAG ATTCATTTGA AGAAGATCCT    300

GAAATTTCCT TAGCTGACTA TTGGAAATGC ACTTCATGCA ATGAAATGAA TCCCCCCCTT    360

CCATCACATT GCAACAGATG TTGGGCCCTT CGTGAGAATT GGCTTCCTGA AGATAAAGGG    420

AAAGATAAAG GGGAAATCTC TGAGAAAGCC AAACTGGAAA ACTCAACACA AGCTGAAGAG    480
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GGCTTTGATG TTCCTGATTG TAAAAAAACT ATAGTGAATG ATTCCAGAGA GTCATGTGTT   540

GAGGAAAATG ATGATAAAAT TACACAAGCT TCACAATCAC AAGAAAGTGA AGACTATTCT   600

CAGCCATCAA CTTCTAGTAG CATTATTTAT AGCAGCCAAG AAGATGTGAA AGAGTTTGAA   660

AGGGAAGAAA CCCAAGACAA AGAAGAGAGT GTGGAATCTA GTTTGCCCCT TAATGCCATT   720

GAACCTTGTG TGATTTGTCA AGGTCGACCT AAAAATGGTT GCATTGTCCA TGGCAAAACA   780

GGACATCTTA TGGCCTGCTT TACATGTGCA AAGAAGCTAA AGAAAAGGAA TAAGCCCTGC   840

CCAGTATGTA GACAACCAAT TCAAATGATT GTGCTAACTT ATTTCCCCTA G   891

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 657 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA   60

GCTTCGGAAC AAGAGACCCT GGACTATTGG AAATGCACTT CATGCAATGA AATGAATCCC   120

CCCCTTCCAT CACATTGCAA CAGATGTTGG GCCCTTCGTG AGAATTGGCT TCCTGAAGAT   180

AAAGGGAAAG ATAAAGGGGA AATCTCTGAG AAAGCCAAAC TGGAAAACTC AACACAAGCT   240

GAAGAGGGCT TTGATGTTCC TGATTGTAAA AAAACTATAG TGAATGATTC CAGAGAGTCA   300

TGTGTTGAGG AAAATGATGA TAAAATTACA CAAGCTTCAC AATCACAAGA AAGTGAAGAC   360

TATTCTCAGC CATCAACTTC TAGTAGCATT ATTTATAGCA GCCAAGAAGA TGTGAAAGAG   420

TTTGAAAGGG AAGAAACCCA AGACAAAGAA GAGAGTGTGG AATCTAGTTT GCCCCTTAAT   480

GCCATTGAAC CTTGTGTGAT TTGTCAAGGT CGACCTAAAA ATGGTTGCAT TGTCCATGGC   540

AAAACAGGAC ATCTTATGGC CTGCTTTACA TGTGCAAAGA AGCTAAAGAA AAGGAATAAG   600

CCCTGCCCAG TATGTAGACA ACCAATTCAA ATGATTGTGC TAACTTATTT CCCCTAG   657

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 966 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA   60

GCTTCGGAAC AAGAGACCCT GGTTAGACCA AAGCCATTGC TTTTGAAGTT ATTAAAGTCT   120

GTTGGTGCAC AAAAAGACAC TTATACTATG AAAGAGGATC TTGATGCTGG TGTAAGTGAA   180

CATTCAGGTG ATTGGTTGGA TCAGGATTCA GTTTCAGATC AGTTTAGTGT AGAATTTGAA   240

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GTTGAATCTC TCGACTCAGA AGATTATAGC CTTAGTGAAG AAGGACAAGA ACTCTCAGAT    300

GAAGATGATG AGGTATATCA AGTTACTGTG TATCAGGCAG GGGAGAGTGA TACAGATTCA    360

TTTGAAGAAG ATCCTGAAAT TTCCTTAGCT GACTATTGGA AATGCACTTC ATGCAATGAA    420

ATGAATCCCC CCCTTCCATC ACATTGCAAC AGATGTTGGG CCCTTCGTGA GAATTGGCTT    480

CCTGAAGATA AAGGGAAAGA TAAAGGGGAA ATCTCTGAGA AAGCCAAACT GGAAAACTCA    540

ACACAAGCTG AAGAGGGCTT TGATGTTCCT GATTGTAAAA AAACTATAGT GAATGATTCC    600

AGAGAGTCAT GTGTTGAGGA AAATGATGAT AAAATTACAC AAGCTTCACA ATCACAAGAA    660

AGTGAAGACT ATTCTCAGCC ATCAACTTCT AGTAGCATTA TTTATAGCAG CCAAGAAGAT    720

GTGAAAGAGT TTGAAAGGGA AGAAACCCAA GACAAGAAG AGAGTGTGGA ATCTAGTTTG     780

CCCCTTAATG CCATTGAACC TTGTGTGATT TGTCAAGGTC GACCTAAAAA TGGTTGCATT    840

GTCCATGGCA AAACAGGACA TCTTATGGCC TGCTTTACAT GTGCAAAGAA GCTAAAGAAA    900

AGGAATAAGC CCTGCCCAGT ATGTAGACAA CCAATTCAAA TGATTGTGCT AACTTATTTC    960

CCCTAG                                                              966
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(2) INFORMATION FOR SEQ ID NO:8:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 399 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA     60

GCTTCGGAAC AAGAGACCCT GGTTAGACAA GAAAGTGAAG ACTATTCTCA GCCATCAACT    120

TCTAGTAGCA TTATTTATAG CAGCCAAGAA GATGTGAAAG AGTTTGAAAG GGAAGAAACC    180

CAAGACAAAG AAGAGAGTGT GGAATCTAGT TTGCCCCTTA ATGCCATTGA ACCTTGTGTG    240

ATTTGTCAAG GTCGACCTAA AAATGGTTGC ATTGTCCATG GCAAAACAGG ACATCTTATG    300

GCCTGCTTTA CATGTGCAAA GAAGCTAAAG AAAAGGAATA AGCCCTGCCC AGTATGTAGA    360

CAACCAATTC AAATGATTGT GCTAACTTAT TTCCCCTAG                           399
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(2) INFORMATION FOR SEQ ID NO:9:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 309 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA     60

GCTTCGGAAC AAGAGACCCT GGTTAGACCA AAGCCATTGC TTTTGAAGTT ATTAAAGTCT    120
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GTTGGTGCAC AAAAAGACAC TTATACTATG AAAGAGGTTC TTTTTTATCT TGGCCAGTAT   180

ATTATGACTA AACGATTATA TGATGAGAAG CAACAACATA TTGTAAATGA TTGTGCTAAC   240

TTATTTCCCC TAGTTGACCT GTCTATAAGA GAATTATATA TTTCTAACTA TATAACCCTA   300

GGAATTTAG   309

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1897 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CACAGATAAG GTTATTTGGG TACCCTCTCG AAAAGTTAAA CCGGACATCG CCCAAAAGGA   60

TGAGGTGACT AAGAAAGATG AGGCGAGCCC TCTTTTTGCA GGCTGGAGGC ACATAGATAA   120

GAGAATTATC ACTCTACATT CATCTTTCTC AAAGATTAAT CTACTTGTGT GTTTTATATT   180

TCATTAGAAT CGGACAGATG TTCAGTGCCA GCACCGGTGG CAGAAAGTAT TAAACCCAGA   240

ACTTAACAAA GGTCCATGGA CTAAAGAGGA GGATCAAAGG GTAATAGAAC ACGTGCAGAA   300

ATACGGTCCA AAGCGCTGGT CGGACATTGC TAAGCATTTG AAGGGAAGGA TTGGAAAACA   360

GTGCAGGGAG AGGTGGCACA ACCATCTGAA TCCAGAAGTG AAGAAACCT CCTGGACAGA   420

AGAGGAAGAT AGAATTATTT ACCAGGCACA CAAGAGACTG GGAAACAGAT GGGCAGAAAT   480

TGCAAAGTTG CTGCCTGGAC GGACTGATAA CGCTGTCAAG AACCACTGGA ATTCCACCAT   540

GCGCCGGAAG GTCGAGCAGG AGGGTTACCC GCAGGAGTCC TCCAAAGCCG GCCCGCCCTC   600

GGCAACCACC GGCTTCCAGA AGAGCAGCCA TCTGATGGCC TTTGCCCACA ACCCACCTGC   660

AGGCCCGCTC CCGGGGGCCG GCCAGGCCCC TCTGGGCAGT GACTACCCCT ACTACCACAT   720

TGCTGAGCCA CAAAATGTCC CTGGTCAGAT CCCATATCCA GTAGCACTGC ATATAAATAT   780

TATCAATGTT CCTCAGCCAG CTGCTGCAGC TATTCAGAGA CACTATACTG ATGAAGACCC   840

TGAGAAAGAA AAACGAATAA AGGAATTAGA GTTGCTACTT ATGTCGACTG AGAATGAACT   900

GAAAGGGCAG CAGGCATTAC CAACACAGAA CCACACAGCA AACTACCCCG GCTGGCACAG   960

CACCACGGTT GCTGACAATA CCAGGACCAG TGGTGACAAT GCGCCTGTTT CCTGTTTGGG   1020

GGAACATCAC CACTGTACTC CATCTCCACC AGTGGATCAT GGTTGCTTAC CTGAGGAAAG   1080

TGCGTCCCCC GCACGGTGCA TGATTGTTCA CCAGAGCAAC ATCCTGGATA ATGTTAAGAA   1140

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TCACGAGAAT CTGAACCTGG ACAACCCTGC ACTAACCTCC ACGCCAGTGT GTGGCCACAA   1260

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GGCTTGACCG AGGGGACTAT AACATGTATA GGCGAAAAGC GGGGTCTCGG TTGTAACGCG   1440

CTTAGGAAGT CCCCTCGAGG TATGGCAGAT ATGCTTTTGC ATAGGGAGGG GGAAATGTAG   1500

TCTTAATCGT AGGTTAACAT GTATATTACC AAATAAGGGA ATCGCCTGAT GCACCAAATA   1560

AGGTATTATA TGATCCCATT GGTGGTGAAG GAGCGACCTG AGGGCATATG GGCGTTAACA   1620

GAACTGTCTG TCCTTGCGTC ATTCCTCATC GGATCATGTA CGCGGCAGAG TATGATTGGA   1680

TAACAGGATG GCACCATTCA TCGTGGCGCA TGCTGATTGG TGCGACTAAG GAGTTGTGTA   1740

ACCCACGAAT GTACTTAAGC TTGTAGTTGC TAACAATAAA GTGCCATTCT ACCTCTCACC   1800

ACATTGGTGT GCACCTGGGT TGATGGCCGG ACCGTCGATT CCCTGACGAC TGCGAACACC   1860

TGAATGAAGC TGAAGGCTTC AGGTACCCTT ACTTGAT   1897

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8082 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTGTTTG GCCGTTTTAG GGTTGTTGG AATTTTTTTT TCGTCTATGT ACTTGTGAAT   60

TATTTCACGT TTGCCATTAC CGGTTCTCCA TAGGGTGATG TTCATTAGCA GTGGTGATAG   120

GTTAATTTTC ACCATCTCTT ATGCGGTTGA ATAGTCACCT CTGAACCACT TTTTCCTCCA   180

GTAACTCCTC TTTCTTCGGA CCTTCTGCAG CCAACCTGAA AGAATAACAA GGAGGTGGCT   240

GGAAACTTGT TTTAAGGAAC CGCCTGTCCT TCCCCCGCTG GAAACCTTGC ACCTCGGACG   300

CTCCTGCTCC TGCCCCCACC TGACCCCCGC CCTCGTTGAC ATCCAGGCGC GATGATCTCT   360

GCTGCCAGTA GAGGGCACAC TTACTTTACT TTCGCAAACC TGAACGCGGG TGCTGCCCAG   420

AGAGGGGGCG GAGGGAAAGA CGCTTTGCAG CAAAATCCAG CATAGCGATT GGTTGCTCCC   480

CGCGTTTGCG GCAAAGGCCT GGAGGCAGGA GTAATTTGCA ATCCTTAAAG CTGAATTGTG   540

CAGTGCATCG GATTTGGAAG CTACTATATT CACTTAACAC TTGAACGCTG AGCTGCAAAC   600

TCAACGGGTA ATAAACCCATC TTGAACAGCG TACATGCTAT ACACACACCC CTTTCCCCCG   660

AATTGTTTTC TCTTTTGGAG GTGGTGGAGG GAGAGAAAAG TTTACTTAAA ATGCCTTTGG   720

GTGAGGGACC AAGGATGAGA AGAATGTTTT TTGTTTTTCA TGCCGTGGAA TAACACAAAA   780

TAAAAAATCC CGAGGGAATA TACATTATAT ATTAAATATA GATCATTTCA GGGAGCAAAC   840

AAATCATGTG TGGGGCTGGG CAACTAGCTG AGTCGAAGCG TAAATAAAAT GTGAATACAC   900

GTTTGCGGGT TACATACAGT GCACTTTCAC TAGTATTCAG AAAAAATTGT GAGTCAGTGA   960

ACTAGGAAAT TAATGCCTGG AAGGCAGCCA AATTTTAATT AGCTCAAGAC TCCCCCCCCC   1020

CCCCAAAAAA AGGCACGGAA GTAATACTCC TCTCCTCTTC TTTGATCAGA ATCGATGCAT   1080

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TTTTTGTGCA TGACCGCATT TCCAATAATA AAAGGGGAAA GAGGACCTGG AAAGGAATTA      1140

AACGTCCGGT TTGTCCGGGG AGGAAAGAGT TAACGGTTTT TTTCACAAGG GTCTCTGCTG      1200

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GTTCGGGTAC CCCCTGCCCC TCCCATATTC TCCCGTCTAG CACCTTTGAT TTCTCCCAAA      1320

CCCGGCAGCC CGAGACTGTT GCAAACCGGC GCCACAGGGC GCAAAGGGGA TTTGTCTCTT      1380

CTGAAACCTG GCTGAGAAAT TGGGAACTCC GTGTGGGAGG CGTGGGGGTG GGACGGTGGG      1440

GTACAGACTG GCAGAGAGCA GGCAACCTCC CTCTCGCCCT AGCCCAGCTC TGGAACAGGC      1500

AGACACATCT CAGGGCTAAA CAGACGCCTC CCGCACGGGG CCCCACGGAA GCCTGAGCAG      1560

GCGGGGCAGG AGGGGCGGTA TCTGCTGCTT TGGCAGCAAA TTGGGGGACT CAGTCTGGGT      1620

GGAAGGTATC CAATCCAGAT AGCTGTGCAT ACATAATGCA TAATACATGA CTCCCCCCAA      1680

CAAATGCAAT GGGAGTTTAT TCATAACGCG CTCTCCAAGT ATACGTGGCA ATGCGTTGCT      1740

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GTTCCGCCTG CGATGATTTA TACTCACAGG ACAAGGATGC GGTTTGTCAA ACAGTACTGC      2040

TACGGAGGAG CAGCAGAGAA AGGGAGAGGG TTTGAGAGGG AGCAAAAGAA AATGGTAGGC      2100

GCGCGTAGTT AATTCATGCG GCTCTCTTAC TCTGTTTACA TCCTAGAGCT AGAGTGCTCG      2160

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TCTCCGCCCA CCGGCCCTTT ATAATGCGAG GGTCTGGACG GCTGAGGACC CCCGAGCTGT      2340

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GCGGGGAGGC TATTCTGCCC ATTTGGGGAC ACTTCCCCGC CGCTGCCAGG ACCCGCTTCT      2820

CTGAAAGGCT CTCCTTGCAG CTGCTTAGAC GCTGGATTTT TTTCGGGTAG TGGAAAACCA      2880

GGTAAGCACC GAAGTCCACT TGCCTTTTAA TTTATTTTTT TATCACTTTA ATGCTGAGAT      2940

GAGTCGAATG CCTAAATAGG GTGTCTTTTC TCCCATTCCT GCGCTATTGA CACTTTTCTC      3000
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AGAGTAGTTA TGGTAACTGG GGCTGGGGTG GGGGGTAATC CAGAACTGGA TCGGGGTAAA    3060

GTGACTTGTC AAGATGGGAG AGGAGAAGGC AGAGGGAAAA CGGGAATGGT TTTTAAGACT    3120

ACCCTTTCGA GATTTCTGCC TTATGAATAT ATTCACGCTG ACTCCCGGCC GGTCGGACAT    3180

TCCTGCTTTA TTGTGTTAAT TGCTCTCTGG GTTTTGGGGG GCTGGGGGTT GCTTTGCGGT    3240

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TGGAGAGGGA AGGTTGGGAG GGGCTGCGGT GCCGGCGGGG GTAGGAGAGC GGCTAGGGCG    4020

CGAGTGGGAA CAGCCGCAGC GGAGGGGCCC CGGCGCGGAG CGGGGTTCAC GCAGCCGCTA    4080

GCGCCCAGGC GCCTCTCGCC TTCTCCTTCA GGTGGCGCAA AACTTTGTGC CTTGGATTTT    4140

GGCAAATTGT TTTCCTCACC GCCACCTCCC GCGGCTTCTT AAGGGCGCCA GGGCCGATTT    4200

CGATTCCTCT GCCGCTGCGG GGCCGACTCC CGGGCTTTGC GCTCCGGGCT CCCGGGGGAG    4260

CGGGGGCTCG GCGGGCACCA AGCCGCTGGT TCACTAAGTG CGTCTCCGAG ATAGCAGGGG    4320

ACTGTCCAAA GGGGGTGAAA GGGTGCTCCC TTTATTCCCC CACCAAGACC ACCCAGCCGC    4380

TTTAGGGGAT AGCTCTGCAA GGGGAGAGGT TCGGGACTGT GGCGCGCACT GCGCGCTGCG    4440

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CCACGGCCGA CCAGCTGGAG ATGGTGACCG AGCTGCTGGG AGGAGACATG GTGAACCAGA    4860

GTTTCATCTG CGACCCGGAC GACGAGACCT TCATCAAAAA CATCATCATC CAGGACTGTA    4920
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TGTGGAGCGG CTTCTCGGCC GCCGCCAAGC TCGTCTCAGA GAAGCTGGCC TCCTACCAGG   4980

CTGCGCGCAA AGACAGCGGC AGCCCGAACC CCGCCCGCGG CCACAGCGTC TGCTCCACCT   5040

CCAGCTTGTA CCTGCAGGAT CTGAGCGCCG CCGCCTCAGA GTGCATCGAC CCCTCGGTGG   5100

TCTTCCCCTA CCCTCTCAAC GACAGCAGCT CGCCCAAGTC CTGCGCCTCG CAAGACTCCA   5160

GCGCCTTCTC TCCGTCCTCG GATTCTCTGC TCTCCTCGAC GGAGTCCTCC CCGCAGGGCA   5220

GCCCCGAGCC CCTGGTGCTC CATGAGGAGA CACCGCCCAC CACCAGCAGC GACTCTGGTA   5280

AGCGAAGCCC GCCCAGGCCT GTCAAAAGTG GGCGGCTGGA TACCTTTCCC ATTTTCATTG   5340

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GGGAGCTCAT CACCTCTGAA ACCTTGGGCT TTAGCGTTTC CTCCCATCCC TTCCCCTTAG   5460

ACTGCCCATG TTTGCAGCCC CCCTCCCCGT TTGTCTCCCA CCCCTCAGGA ATTTCATTTA   5520

GGTTTTTAAA CCTTCTGGCT TATCTTACAA CTCAATCCAC TTCTTCTTAC CTCCCGTTAA   5580

CATTTTAATT GCCCTGGGGC GGGGTGGCAG GGAGTGTATG AATGAGGATA AGAGAGGATT   5640

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TGCACACCAC CATGCCAGGC TAATTTTTGT AATTTTAGTA GAGATGGGGT TTCATCGTGT   6420

TGGCCAGGAT GGTCTCTCCT GACCTCACGA TCCGCCCACC TCGGCCTCCC AAAGTGCTGG   6480

GATTACAGGT GTGAGCCAGG GCACCAGGCT TAGATGTGGC TCTTTGGGGA GATAATTTTG   6540

TCCAGAGACC TTTCTAACGT ATTCATGCCT TGTATTTGTA CAGCATTAAT CTGGTAATTG   6600

ATTATTTTAA TGTAACCTTG CTAAAGGAGT GATTTCTATT TCCTTTCTTA AAGAGGAGGA   6660

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GGTCCTCAAG AGGTGCCACG TCTCCACACA TCAGCACAAC TACGCAGCGC CTCCCTCCAC   6840
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TCGGAAGGAC TATCCTGCTG CCAAGAGGGT CAAGTTGGAC AGTGTCAGAG TCCTGAGACA    6900

GATCAGCAAC AACCGAAAAT GCACCAGCCC CAGGTCCTCG GACACCGAGG AGAATGTCAA    6960

GAGGCGAACA CACAACGTCT TGGAGCGCCA GAGGAGGAAC GAGCTAAAAC GGAGCTTTTT    7020

TGCCCTGCGT GACCAGATCC CGGAGTTGGA AAACAATGAA AAGGCCCCCA AGGTAGTTAT    7080

CCTTAAAAAA GCCACAGCAT ACATCCTGTC CGTCCAAGCA GAGGAGCAAA AGCTCATTTC    7140

TGAAGAGGAC TTGTTGCGGA AACGACGAGA ACAGTTGAAA CACAAACTTG AACAGCTACG    7200

GAACTCTTGT GCGTAAGGAA AAGTAAGGAA AACGATTCCT TCTAACAGAA ATGTCCTGAG    7260

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AAAGAACTTT TTTATGCTTA CCATCTTTTT TTTTTCTTTA ACAGATTGT ATTTAAGAAT     7440

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CCTAGTATTA TAGGTACTAT AAACCCTAAT TTTTTTTATT TAAGTACATT TTGCTTTTTA    7620

AAGTTGATTT TTTTCTATTG TTTTTAGAAA AAATAAAATA ACTGGCAAAT ATATCATTGA    7680

GCCAAATCTT AAGTTGTGAA TGTTTTGTTT CGTTTCTTCC CCCTCCCAAC CACCACCATC    7740

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GAGAAGGTGA GAGGTAGGCA AAGGAGATAC AAGAGGTCAA AGGTAGCAGT TAAGTACACA    7980

AAGAGGCATA AGGACTGGGG AGTTGGGAGG AAGGTGAGGA AGAAACTCCT GTTACTTTAG    8040

TTAACCAGTG CCAGTCCCCT GCTCACTCCA AACCCAGGAA TT                        8082
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(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 4480 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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AGGGTTACAC GTCTTAACTC AGAGTTGCAA CAGGCTTGAA CAAGCCCAGG CACGCCCAGA      60

TACCTAGGGC CGAGTCACCG TTAAAACTAA CAGACCATAA AAGGAAAGGA ATACAGAACA     120

GACTAGGAGT ACCGGATCTG ACTCACAGGC CACCTGGCAG GAAGAGATAA GCCCCAGCCC     180

CCGACATTCA GGACGTCCCA GCCCGCACGT ACTCTTACCA TGTTACAACC TCATTCGAAT     240

ATGATTCAAA CCTGCCAATG TGTGTAGCTA TACCTTATCA CCTCATCTTG TGAAATAACC     300

AATCATATGT GAACATGTCT ATATGCTTCG TTTAAATCCA CCAATCCCCG TAACTATGCA     360
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TCTGCTTCTG TACGCCCGCT TCTGCTTCCC CAAACCCTAT AAAAGCCCCA TGCTAGAGCT    420

GTTGGGCGCG CAAGTCCTCC GAAGAGACTG TGTGCCCGCA GGTACCTGTG TTTTCCAATA    480

AACCCTCTTG CTGATTGCAT CCGAGTGGCC TCGGCTCGGT CATTGGGCGC TTGGGGGTCT    540

CCTCCTGAGG GAAAGGTCCT CTCCGGAGGT CTTTTCATTT TGGGGGCTCG TCCGGGATCT    600

GGAGATCCTC CGCCCAGAGA TCACCGACCA CCCACCGGGA GGTAAGCCGG CCGGCATCTG    660

TCGTGTCTTG CCCTGTCTTG TCTTGTCTTG TCCTGTGCGC GTGTTCAGTT CGTCTCAGTT    720

TTGGACTCAG ATCTGGGTTT TGGTCGAAGG AGAAGGCCCA GGGCTTCGGT TTCTCAGGGT    780

TCAGGACCCT CAGCGCCTCC GTTTGGGCGG GTCAGAGAAG GAGCTGACGA GCTCGGACTT    840

CTCCCCCCGC AGCCCTGGAA GACGTTCCAA GGGTGTCTGG AGCCCGGTTC TTTGGGGCTC    900

AGCCCGTATC GGAGGGATAC GTGGTTTTGG TTGGAGGAGA GGGTCCAGGA CCCTCGGCAC    960

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AAGAGTATAG TGCCATGCGG GACCAGTACA TGCGCACAGG GGAGGGCTTC CTCTGTGTAT   1500

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GGGTGAAAGA TTCAGATGAT GTGCCAATGG TGCTGGTGGG CAACAAGTGT GACCTGGCCG   1620

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TGAGCTGCAA GTGTGTGCTG TCCTGACACC AGGTTAAGGA CCTGATTTTC CGCCAGAAGC   1860

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CCCTTATAGA TCTCCTGTTG GAAGAACCTC CTCCGTACCC TGTACCTACA GCCCCGCCAA   2160

GAGAAGAGGA AGTGGAGCCG CCTGCTAGAC CTCGACTCGA GGCGGCCCCT TCCCCTGTGG   2220

CTGGAAGACT TCGGGGACGA CGCGAGGTGG CGCCAGACTC CACCTCCCAG GCCTTTCCGC   2280
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TTAGACAAGG GGCTGGCGGC CAGATACAAT ACTGGCCATT CTCAGCGGCC GACATATATA    2340

ACTGGAAACA ACACAACCCC CCCTTTTCTA AGGATCCGGT GGCTCTCACC AACCAGATAG    2400

AATCTGTCTT GCTTACCCAT CAGCCCACTT GGGATGATAT ACAGCAACTT TTACAGGCCC    2460

TCCTGACCTC TGAAGAGAAG CAGAGAGTGC TCTTAGAGGC CAGGAAACAT GTTTTGGGGG    2520

ACAATGGACG CCCCACCTTG CTCCCGAAAG AGATCGATGA TGCATTCCCA CTTACAAGAC    2580

CTGATTGGGA TTTCACCACG GCTAAAGGTA GGAGACACCT ACGCCTTTAT CGCCAGTTGC    2640

TCCTAGCGGG TCTCCGAGGG GCGGCACGAC GCCCCACCAA TTTGGCTCAG GTAAAACAAG    2700

TGGTACAAGA GGCTGCGGAG ACTCCCTCAG CCTTCCTAGA GAGACTTAAG GAAGCTTATC    2760

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GGTATACACT GCAGGATTTA CTTAAGGAGG CAGAAAGAAT TTATAACAAG AGAGAGACAC    2940

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TAAGGTGACC CATTCCTTCT TACATGTTCC GGACTGCCCA TACCCTCTGC TGGGCCGTGA    3480

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TCTGAAGGAT GCTTTCTTCT GCCTCCGGCT GAGCCCAGAA AGCCAGCCCT TATTTGCTTT    4080

TGAGTGGAAA GACTCTGAAA TGGGGCTTTC GGGACAGTTG ACTTGGACAA GGTTACCACA    4140

GGGTTTCAAA AACAGCCCAA CGCTCTTTGA TGAGGCCTTA CACCGGGACT GGCTGACTT     4200
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TCGAGTCCAG CATCCCACTC TTATACTTCT TCAGTTTGTT GATGACCTTC TTCTAGGGGC   4260

CACTTCTGAG ACAGCATGCC ACCAGGGAAC AGAATCCCTC TTGCAGACTT TGGGGCGATT   4320

GGGCTATCGA GCTTCTGCCA GAAAGGCTCA AATTTGCCAG ACCCAGGTTA CTTATTTAGG   4380

CTATCAACTA AGGGATGGAC AGCGATGGCT GACTCCGGCT AGGAAACAGA CCGTGGCCAA   4440

CATCCCAGCC CCAAGAAATG GCCGACAGCT ACGGGAATTC   4480

(2) INFORMATION FOR SEQ ID NO:13:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 565 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTGAGTAGT GCGCGAGCAA AATTTAAGCT ACAACAAGGC AAGGCTTGGC CGACAATTGC   60

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ACGCGTATCT GAGGGGACTA GGGTGTGTTT AGGCGAAAAG CGGGGCTTCG GTTGTACGCG   180

GTTAGGAGTC CCCTCAGGAT ATAGTAGTTT CGCTTTTGCA TAGGGAAGGG GAAATGTAGT   240

CTTATGCAAT ACTCTTGTAG TCTTGCAACA TGCTTATGTA ACGATGAGTT AGCAACATGC   300

CTTACAAGGA GAGAAAAAGC ACCGTGCATG CCGATTGGTG GAAGTAAGGT GGTACGATCG   360

TGCCTTATTA GGAAGGCAAC AGACGGGTCT GACATGGATT GGACGAACCA CCGAATTCCG   420

CATTGCAGAG ATATTGTATT TAAGTGCCTA GCTCGATACA ATAAACGCCA TTTGACCATT   480

CACCACATTG GTGTGCACCT GGGTTGATGG CCGGACCGTT GATTCCCTGA CGACTACGAG   540

CACCTGCATG AAGCAGAAGG CTTCA   565

(2) INFORMATION FOR SEQ ID NO:14:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1804 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGATCCTCAG GGGTAACACC TTTTGGAGGT GGGCATCTTC CTCATTCTCA GTGGTGCCAA   60

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AAGCTAGAAC TACAGAAGAC CTTTCATTTA AGAAGGGTGA AAAATTTCAA ATAATTAACA   180

ATACAGAAGG AGACTGGTGG GAAGCAAGAT CAATCACTAC AGGAAAGAAT GGTTATATCC   240

TGAGCAGTTA TGTAGCGCCT GCAGATTCCA TTCAGGCAGA AGAATGGTAT TTTGGCAAAA   300

TGGGGAGAAA AGATGCTGAA AGATTACTTC TGAATCCTGG AAATTAATGA GGTATTTTCT   360

TAGGAAGAGA GAGTGAAATG GCTGGGTGCA GTGGCTCATG CCTGTAATCC CAGCACTTTG   420

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CCTGTAATCC CAGCTACTCA GGAGGCTGAG GCAGCAGAAT CACTTGAACC TGGGAGGCGG      600

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TACTAAAGGT GCTTATTCCC TCTCTATTCG TGATTGGGAT GAGGTAAGGG GTGACAATGT      780

GAAACACCAC AAAATTAGGA AACTTGACAA TGGTAGATAC TATATCACAA CCAGAGAACA      840

ACTTGATACT CTGCAGAAAT TGGCAAAACA CTACACAGAA CATGCTGATG GTTTATGCCA      900

CAAGTTAACA ACTGTGTGTC CAACTGTGAA ACCTCAGATT CAAGGTCTAG CAAAAGATGC      960

TTGGGAAATC CCTTGATAAT CTTTGCGACT AGAGGTTAAA CTAGGACAAG GATGTTTTGG     1020

CAAAGTGTGG ATGGGAATAT GGAATGGAAC CACAAAAGTA GCAATCAAAA CACTAAAACC     1080

AGGTACAATG ATGCCAGAAG CTTTTCTTCA AGAAGCTCAG GTAATGAAAA AAATAAGACA     1140

TGGTAAACTT GTTCCACTAT ATGCTGTTGT TTCTGAAGAG CCAATTTACA TTGTCACTGA     1200

ATTGATGTCA AAAGGAAGCT TATTCAATTT CCTTAAGGAA GGAGATGGAA AGTATTTGAA     1260

GCTTCCACAA ATGGTTGATA TGCCTGCTCA GATTGCTGAT GGTATGGCAT ATATTAAAAG     1320

AATGAACTAT ATTCACCGAG ATCTCTGGGC TGCTAATATT CTTGTAGGAG AAAATCTTCT     1380

GTGCAAAATA GCAGATTTTG GTTTAGCAAG GTTAATTGAA GACAATGAAT ACACATCAAG     1440

ACAAGGTGCA GAATTTCCAA TCAAATGGAC AGCTCCTGAA GTTGCACTGT ATGGTGGGTT     1500

TACAATAAAG TCTGGTGTCT GCTCATTTGG AATTCTACAG ACAGAACTGG TAACAAAGGG     1560

CAGAGTGCCA TATCCAGGTA TGGTGAACCA TGAAATACTG GAACAGGTGG AGCGAGGATA     1620

CAGGATGCCT TGCCCTCAGG CTGTCCAGA ATCCCTCCAT GAATTGATGA ATCTGTGTTG     1680

GAAGAAGGAC CCTGATGAAA GACCAACATT TGAATATGTT CAGTCCTTCT TGGGAGACTA     1740

CTTCACTGCT ACAGAGCCAT AGTACCAGCC AGGAGAAAAC TTCTAATTCA AGTAGCCTAT     1800

TTTA                                                                 1804
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Claims

1.   A cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, which cellular immunogen comprises host cells which have been transfected with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

2.   An immunogen according to claim 1 wherein the transgene comprises

                    wild-type or mutant retroviral oncogene DNA; or

                    wild-type or mutant proto-oncogene DNA of a species different from the host species.

3.   An immunogen according to claim 2 wherein the transfected cells are non-dividing.

4.   An immunogen according to claim 2 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

5.   An immunogen according to claim 4 wherein the mutant DNA is nontransforming.

6.   An immunogen according to claim 5 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

7. A cellular immunogen according to claim 6 wherein the host cells have been transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

8. An immunogen according to claim 1 wherein the host cells have been transfected with a transgene cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

9. An immunogen according to claim 1 wherein the cells comprise fibroblasts.

10. A method for preparing a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, the method comprising:

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

11. A method according to claim 11 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

12. A method according to claim 11 wherein the transfected cells are non-dividing.

13. A method according to claim 11 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

14. A method according to claim 13 wherein the mutant DNA is nontransforming.

15. A method according to claim 14 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

16. A method according to claim 15 wherein the host cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

17. A method according to claim 11 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-erbB-2, MDM-2, c-myc, c-myb, c-ras, c-src and c-yes.

18. A method according to claim 1 wherein the excised cells comprise fibroblasts.

19. A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene comprising
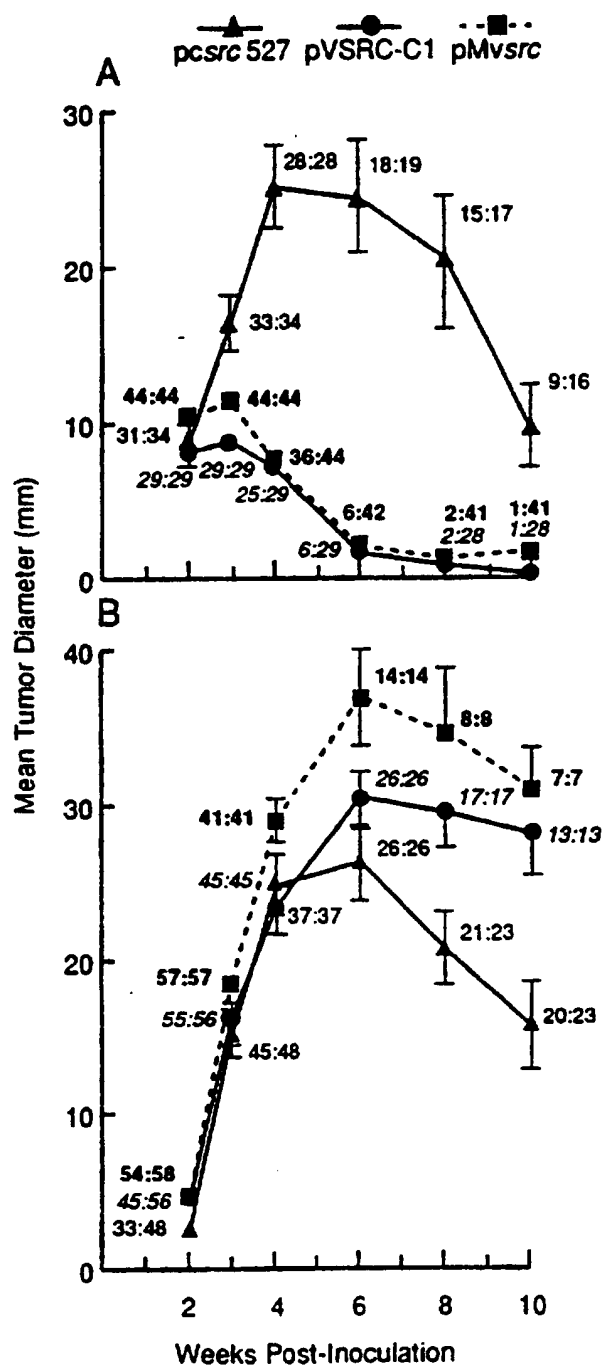(a) excising cells from the host;
(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of

the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

20. A method according to claim 19 wherein the transgene comprises

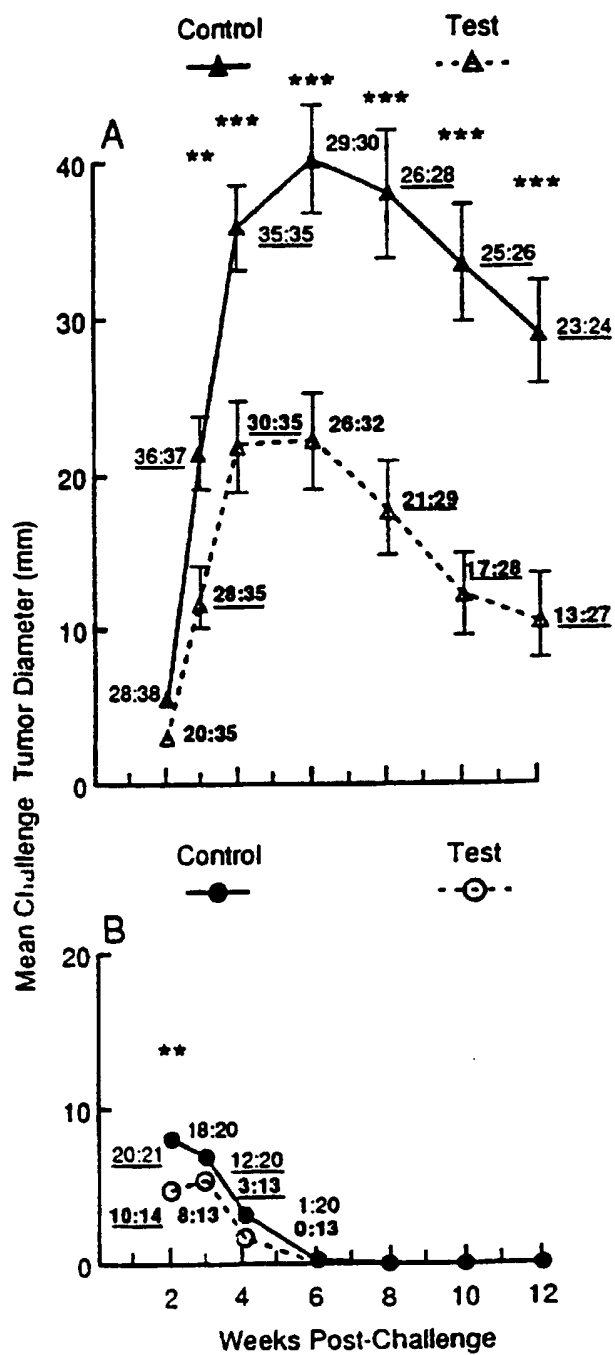wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

21. A method according to claim 20 wherein the transfected cells are rendered non-dividing prior to return to the body of the host.

22. A method according to claim 20 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

23. A method according to claim 22 wherein the mutant DNA is nontransforming.

24. A method according to claim 23 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

25. A method according to claim 24 wherein the host cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

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26. A method according to claim 19 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

27. A method according to claim 19 wherein the excised host cells comprise fibroblasts.

28. A method of vaccinating a host against disease associated with the overexpression of a targeted proto-oncogene comprising

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least transgene and a strong promoter to drive the expression of the transgene in the transfected cells, wherein the transgene comprises

(1) wild-type or mutant cognate retroviral oncogene DNA; or

(2) wild-type or mutant cognate proto-oncogene DNA of a species different from the host species;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

FIG. 1

FIG. 2

FIG. 3

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)　: A01K 63/00; A61K 39/00, 39/38, 48/00; C12N 5/00, 15/00
US CL　: 424/93.21, 184.1; 435/172.1, 240.1
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :　424/93.21, 184.1; 435/172.1, 240.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS
Search terms: oncogene/transfection/vaccine/proto-oncogene

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MALONE et al. Cationic liposome-mediated RNA transfection. Proc. Natl. Acad. Sci. USA. August 1989, Vol. 86, pages 6077-6081, see entire document	1-18
Y,E	US 5,593,972 (WEINER et al.) 14 January 1997, see entire document.	1-28
Y	FENDLY et al. The extracellular domain of HER2/neu is a potential immunogen for active specific immunotherapy of breast cancer. J. Biol. Response Mod. October 1990, Vol. 9, No. 5, pages 449-455, see entire document.	1-28

|X| Further documents are listed in the continuation of Box C.　　[ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 1997	1 2 MAY 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer RAY F. EBERT
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INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FELGNER et al. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA. November 1987, Vol. 84, pages 7413-7417, see entire document.	1-28
Y	FENTON et al. Cytotoxic T-cell response and In Vivo protection against tumor cells harboring activated ras proto-oncogenes. J. Natl. Cancer Inst. 18 August 1993, Vol. 85, No. 16, pages 1294-1302, see entire document.	1-28
Y	MCCABE et al. Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T lymphocyte responses. Cancer Res. 15 April 1995, Vol. 55, pages 1741-1747, see entire document.	1-28
Y	TEMIN, H.M. Overview of biological effects of addition of DNA molecules to cells. J. Med. Virol. May 1990, Vol. 31, pages 13-17, see entire document.	1-28
Y	CONRY et al. Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res. 01 April 1995, Vol. 55, pages 1397-1400, see entire document.	1-28

CORRECTED VERSION*

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES

(57) Abstract

A cellular immunogen is provided for immunizing a host against the effects of the product of a target proto-oncogene, where the overexpression of the target proto-oncogene is associated with a malignancy. The cellular immunogen comprises host cells which have been transfected with at least one transgene construct comprising a transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells. The transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene. The transgene may comprise, for example, wild-type or mutant retroviral oncogene DNA cognate to the target proto-oncogene; or wild-type or mutant proto-oncogene DNA of a species different from the host species. The cellular immunogen may be prepared from biopsied host cells, e.g. skin fibroblasts, which are stably or transiently transfected with the transgene construct containing the cognate transgene. The host cells transfected with the cognate transgene construct, are then returned to the body of the host to obtain expression of the cognate transgene in the host.

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"CELLULAR IMMUNOGENS USEFUL AS CANCER VACCINES"

Cross-Reference to Related Application

Priority from U.S. provisional patent application No. 60/010,262, filed January 19, 1996 is claimed.

5

Field of the Invention

The invention relates to the field of cancer vaccination and immunotherapy.

Background of the Invention

A current goal of cancer research is the identification of host
10   factors that either predispose to tumor formation or serve to enhance tumor growth.

Genes that confer the ability to convert cells to a tumorigenic state are known as **oncogenes**. The transforming ability of a number of retroviruses has been localized in individual viral oncogenes (generally v-*onc*).
15   Cellular oncogenes (generally c-*onc*) present in many species are related to viral oncogenes. It is generally believed that retroviral oncogenes may represent escaped and/or partially metamorphosed cellular genes that are incorporated into the genomes of transmissible, infectious agents, the retroviruses.

Some c-*onc* genes intrinsically lack oncogenic properties, but may
20   be converted by mutation into oncogenes whose transforming activity reflects the acquisition of new properties, or loss of old properties. Amino acid

substitution can convert a cellular proto-oncogene into an oncogene. For example, each of the members of the c-*ras* proto-oncogene family (H-*ras*, N-*ras* and K-*ras*) can give rise to a transforming oncogene by a single base mutation.

Other c-*onc* genes may be functionally indistinguishable from the
5   corresponding v-*onc*, but are oncogenic because they are expressed in much greater amounts or in inappropriate cell types. These oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized example of this type of proto-oncogene is c-*myc*. Changes in *MYC* protein sequence do not appear to be essential for
10   oncogenicity. Overexpression or altered regulation is responsible for the oncogenic phenotype. Activation of c-*myc* appears to stem from insertion of a retroviral genome within or near the c-*myc* gene, or translocation to a new environment. A common feature in the translocated loci is an increase in the level of c-*myc* expression.

15   Gene amplification provides another mechanism by which oncogene expression may be increased. Many tumor cell lines have visible regions of chromosomal amplification. For example, a 20-fold c-*myc* amplification has been observed in certain human leukemia and lung carcinoma lines. The related oncogene N-*myc* is five to one thousand fold amplified in
20   human neuroblastoma and retinoblastoma. In human acute myeloid leukemia and colon carcinoma lines, the proto-oncogene c-*myb* is amplified five to ten fold. While established cell lines are prone to amplify genes, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular oncogenes in many independent tumors of the same type, strengthens
25   the correlation between increased expression and tumor growth.

Immunity has been successfully induced against tumor formation by inoculation with DNA constructs containing v-*onc* genes, or by inoculation with v-*onc* proteins or peptides. A series of reports describe a form of "homologous" challenge in which an animal test subject is inoculated with either
30   v-*src* oncoprotein or DNA constructs containing the v-*src* gene. Protective immunity was induced against tumor formation by subsequent challenge with v-

src DNA or *v-src*-induced tumor cells. *See*, Kuzumaki *et al.*, *JNCI* (1988), 80:959-962; Wisner *et al.*, *J. Virol.* (1991), 65:7020-7024; Halpern *et al.*, *Virology* (1993), 197:480-484; Taylor *et al.*, *Virology* (1994), 205:569-573; Plachy *et al.*, *Immunogenetics* (1994), 40:257-265. A challenge is said to be "homologous" where reactivity to the product of a targeted gene is induced by immunization with the same gene, the corresponding gene product thereof, or fragment of the gene product. A challenge is "heterologous" where reactivity to the product of a targeted gene is induced by immunization with a different gene, gene product or fragment thereof.

WO 92/14756 (1992) describes synthetic peptides and oncoprotein fragments which are capable of eliciting T cellular immunity, for use in cancer vaccines. The peptides and fragments have a point mutation or translocation as compared to the corresponding fragment of the proto-oncogene. The aim is to induce immunoreactivity against the mutated proto-oncogene, not the wild-type proto-oncogene. WO 92/14756 thus relates to a form of homologous challenge.

EP 119,702 (1984) describes synthetic peptides having an amino acid sequence corresponding to a determinant of an oncoprotein encoded by an oncogenic virus, which determinant is vicinal to an active site of the oncoprotein. The active site is a region of the oncoprotein required for oncoprotein function, e.g., catalysis of phosphorylation. The peptides may be used to immunize hosts to elicit antibodies to the oncoprotein active site. EP 119,702 is thus directed to a form of homologous challenge.

The protein product encoded by a proto-oncogene constitutes a self antigen and, depending on the pattern of its endogenous expression, would be tolerogenic at the level of T cell recognition of the self peptides of this product. Thus, vaccination against cancers which derive from proto-oncogene overexpression is problematic.

Recent attempts have been made to induce immunity *in vitro* or *in vivo* to the product of the HER-2/*neu* proto-oncogene. The proto-oncogene encodes a 185-kDa transmembrane protein. The HER-2/*neu* proto-oncogene is overexpressed in certain cancers, most notably breast cancer. In each report

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discussed below, the immunogen selected to induce immunity comprised a
purified peptide of the p185$^{HER-2/neu}$ protein, and not a cellular immunogen.

Disis *et al.*, *Cancer Res.* (1994) 54:16-20 identified several
breast cancer patients with antibody immunity and CD4+ helper/inducer T-cell
5   immunity responses to p185$^{HER-2/neu}$ protein. Antibodies to p185$^{HER-2/neu}$ were
identified in eleven of twenty premenopausal breast cancer patients. It was
assumed prior to this work that patients would be immunologically tolerant to
HER-2/*neu* as a self-protein and that immunity would be difficult to generate.

Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076 constructed
10   synthetic peptides identical to p185$^{HER-2/neu}$ protein segments with amino acid
motifs similar to the published motif for HLA-A2.1-binding peptides. Out of
four peptides synthesized, two were shown to elicit peptide-specific cytotoxic
T-lymphocytes by primary *in vitro* immunization in a culture system using
peripheral blood lymphocytes from a normal individual homozygous for HLA-
15   A2. Thus, it was concluded that the p185$^{HER-2/neu}$ proto-oncogene protein
contains immunogenic epitopes capable of generating human CD8^{+} cytotoxic T-
lymphocytes.

The cytotoxic T cells elicited in the latter report were not,
however, shown to recognize tumor cells, but only targets that bound the
20   synthesized peptides. Other work (Dahl *et al.*, *J. Immunol.* (1996), 157:239-
246) has demonstrated that cytotoxic cells may recognize targets that bind
peptide but fail to recognize targets that endogenously synthesize peptide. It is
thus unclear whether the cytotoxic cells elicited by Disis *et al.* would be capable
of recognizing tumor cells. In any event, no protection against tumor growth
25   was demonstrated by Disis *et al.*

Peoples *et al.*, *Proc. Natl. Acad. Sci. USA* (1995), 92:432-436,
report the identification of antigenic peptides presented on the surface of ovarian
and breast cancer cells by HLA class 1 molecules and recognized by tumor-
specific cytotoxic T lymphocytes. Both HLA-A2-restricted breast and ovarian
30   tumor-specific cytotoxic T lymphocytes recognized shared antigenic peptides.

T cells sensitized against a nine-amino acid sequence of one of the peptides demonstrated significant recognition of HLA-A2 HER2/*neu* tumors.

It remains unclear whether Peoples *et al.* have successfully attacked proto-oncogene-encoded self, as the immunizing peptide which is expressed in the tumor cells contained an isoleucine at position 2, whereas the peptide expressed in normal tissue contains valine residue at this position. Moreover, although stimulation of T cells occurred *in vitro*, this stimulation does not represent a true primary immune response insofar as the starting T cell population represented tumor infiltrating lymphocytes.

The research accounts of Disis *et al.* and Peoples *et al.* required a form of *in vitro* stimulation, either priming as described by Disis *et al.*, or restimulation as described by Peoples *et al.* The *in vitro* protocols of Disis *et al.* and Peoples *et al.* require a mutant cell line to aid in selection of the peptide which will serve to induce reactivity. Non-mutant, peptide antigen-presenting cells have their HLA class I molecules already loaded with endogenous peptides, a phenomenon which precludes exogenous loading from without. The value of the mutant lines is that they lack the TAP genes (encoding the transporters associated with antigen presentation). Class I binding of internally-derived peptides is significantly lowered, and "empty" class I molecules are present on the cell surface and available for binding of exogenously added peptides. This availability of peptide binding sites on membrane-bound class I allows examination of whether a given peptide will (i) even bind to class 1, and (ii) function as a target in cytotoxic T cell assays. However, the need for a mutant cell line for deduction of candidate immunizing peptide sequences limits the usefulness of peptide-based immunization schemes.

Fendly *et al.*, *J. Biol. Response Modifiers* (1990), 9:449-455 present an account of a polypeptide-based immunotherapy. Purified polypeptide corresponding to the extracellular domain of the p185[HER-2/neu] protein was obtained from a transfected cell line. The purified peptide was employed in the immunization of guinea pigs. The immunized animals developed a cellular immune response, as monitored by delayed-type hypersensitivity. Antisera

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derived from immunized animals specifically inhibited the *in vitro* growth of human breast tumor cells overexpressing p185$^{HER-2/neu}$. There is no indication by Fendly *et al.* of induction of self versus non-self reactivity. It is likely that the guinea pigs were chiefly responding to non-self determinants (as defined in terms of the guinea pig host) on the human polypeptide immunogen.

The use of peptides for immunization is of necessity limited to immunization with a single haplotype. There are approximately thirty HLA types in man. In each case of peptide immunization, one must be careful to select peptides which match the host HLA type. The selected peptide must be immunogenic in the host and be capable of presentation to host immune system cells.

What is needed is an immunization method for immunizing humans and animals against self-encoded proto-oncogenes which are associated with the development of cancer, which dispenses with the need for isolating immunogenic, HLA host-matched peptides for immunization.

Summary of the Invention

It is an object of the invention to induce reactivity to self-determinants of the product of an overexpressed proto-oncogene.

It is an object of the invention to provide for a form of therapy or prophylaxis based upon the capacity to induce immune reactivity to proto--oncogene-encoded self as overexpressed in tumor cells.

It is an object of the invention to provide a cellular immunogen for use in immunization against self proto-oncogene determinants.

It is an object of the invention to provide for a method for vaccinating a host against disease associated with the overexpression of a proto-oncogene.

These and other objects will be apparent from the following disclosure.

A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene is provided. The method comprises:

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

According to one principal embodiment of the invention, the transgene comprises wild-type or mutant retroviral oncogene DNA. According to another principal embodiment of the invention, the transgene comprises wild-type or mutant proto-oncogene DNA of a species different from the host species. Where the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA, the mutant DNA is preferably nontransforming. The mutant DNA preferably comprises a deletion mutation in a region of the DNA which is essential for transformation. Preferably, the host cells are transfected with a plurality, most preferably at least five, different transgene constructs, each construct encoding a different deletion mutation.

In one preferred embodiment of the invention, the mutant DNA has at least about 75% homology, more preferably at least about 80% homology, most preferably at least about 90% homology, with the corresponding wild-type oncogene or proto-oncogene DNA.

The invention is further directed to a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which is associated with a cancer. The cellular

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immunogen comprises the host cells which have been transfected with at least one transgene construct, as described above.

The invention is also directed to a method of preparing the cellular immunogen, by (a) excising cells from the host, and (b) transfecting the excised cells with at least one transgene construct, as described above.

The cells transfected with the transgene are preferably rendered non-dividing prior to return to the body of the host.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (*i.e.*, is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human c-*myc* gene is the cognate gene to the mouse c-*myc* gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode proteins which are functionally equivalent.

By "homology" is meant the degree of sequence similarity between two different amino acid sequences, as that degree of sequence similarity is derived by the FASTA program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

The word "transfection" is meant to have its ordinary meaning, that is, the introduction of foreign DNA into eukaryotic cells.

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By "transgene" is meant a foreign gene that is introduced into one or more host cells.

By "transgene construct" is meant DNA containing a transgene and additional regulatory DNA, such as promoter elements, necessary for the expression of the transgene in the host cells.

Description of the Figures

Figs. 1A and 1B are plots of the mean tumor diameter over time following subcutaneous wing web inoculation of 1-day-old line TK (Fig. 1A) and line SC (Fig. 1B) chickens with 100 μg of tumorigenic plasmids pcsrc527 (—▲—), pVSRC-C1 (—●—) or pMvsrc (--■--). The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated was computed as the sum of the diameters of the primary tumors divided by the number of chickens surviving to that point. The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for pcsrc527, italics for pVSRC-C1, bold typeface for pMVsrc). Error bars (unless obscured by the symbol) indicate standard error.

Figs. 2A and 2B are plots of the growth of challenge (wing web) tumors in test and control line TK chickens under conditions of (i) priming and homologous challenge with plasmid pcsrc527 (Fig.2A: --△--, test; --▲--, control), or (ii) priming and homologous challenge with plasmid pVSRC-C1 (Fig. 2B: --○--, test; --●--, control). Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge diameter was computed as in Figs. 1A and 1B. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). The statistical

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comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where $p < 0.05$. Error bars indicate standard error.

5   Figs. 3A and 3B are plots of the growth of challenge (wing web) tumors in TK chickens under conditions of (i) priming with plasmid pVSRC-C1 and heterologous challenge with plasmid pcsrc527 (Fig. 3A: $--\triangle--$, test; $--\blacktriangle--$, control) or (ii) priming with pcsrc527 and heterologous challenge with pVSRC-C1 (Fig. 3B: $--\bigcirc--$, test; $--\bullet--$, control). Test chickens were

10   primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as in Figs. 1A and 1B. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated (standard typeface for control group, bold

15   typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described for Figs. 2A and 2B. [*($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, $p < 0.05$. Error bars indicate standard error.

20   **Detailed Description of the Invention**

A vaccination strategy is provided to prevent development of cancers. The vaccination method may be carried out on a subject at risk for a particular cancer, but before the development of the cancer. The practice of the invention may serve for the immunoprevention of prevalent human cancers,

25   such as colon carcinoma, breast carcinoma, and various lymphomas whose progress is accompanied by the overexpression of a cellular proto-oncogene.

The vaccination strategy of the present invention relies on the induction of an immune response that targets tumor cells by virtue of the recognition of the proto-oncogene-specific antigenicity. The aim of the vaccine

30   protocol is to induce reactivity to self-determinants of an overexpressed proto-

oncogene product. The strategy exploits the structural relatedness between the product of the cellular proto-oncogene and that of the product of genes cognate to the target proto-oncogene. The cognate gene may comprise a wild-type or mutant cognate retroviral oncogene or a wild-type or mutant proto-oncogene of a species different from the host species. The starting point of the vaccine strategy is the high degree of primary sequence homology that exists between the protein product of a targeted proto-oncogene and that of its cognate retroviral oncogene, or between the proto-oncogene product and the product of a cognate proto-oncogene from a different species. However, in contrast to other proposed vaccine strategies, the present invention is not based on the immune recognition of a determinant defined by a cancer specific mutation.

For those tumors showing proto-oncogene overexpression, this sequence homology permits application of the following strategy, which can be employed either prophylactically or therapeutically under conditions of cell-surface expression, or other forms of adjuvanicity, as chosen to enhance immunogenicity: (a) immunization of host biopsied cells with a DNA construct comprising a transgene cognate to the target proto-oncogene, which transgene encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene; (b) return of the transfected cells to the body of the host to obtain expression of the transgene in the host, and thus immunity against the proto-oncogene product. The invention relies on the targeting of a self-determinant found on an overexpressed or overabundant proto-oncogene-encoded product. The foreign peptide elements of the immunizing oncogene product will trigger peripheral lymphocytes exhibiting a weak cross reactivity for the self peptides of the targeted proto-oncogene product. Although such self peptides would be present in normal cells expressing the proto-oncogene, targeting of the tumor cells is favored in view of their overexpression of the proto-oncogene.

The immune strategy exploits the antigenicity of two alternative types of determinants: (1) tumor-associated antigenic determinant(s) induced as a consequence of the activity of the oncogene product, e.g., an enzymatic

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modification of a cellular protein effected by the oncogene product, or (2) tumor associated antigenic determinant(s) intrinsic to the oncogene-encoded product itself. The difficulty in exploiting the first alternative by traditional means, i.e., antigen purification, is that at present little or no systematic information exists

5   bearing on the properties of an antigen that, though oncogene-induced, is not oncogene-encoded. This situation makes purification of any such antigen problematic. However, this problem is obviated from the outset by the present invention which utilizes biopsied cells which, as transfected in culture by the cognate retroviral oncogene, would express the relevant antigenicity.

10   In terms of exploiting the second alternative, that of an antigenicity intrinsic to the proto-oncogene product, a relevant consideration is that the protocol of immunization according to the present invention primes the host to determinants of the oncogene product itself. A consequence of this immunization is induction of T-cell reactivity to the divergent, i.e foreign,

15   peptide determinants of the retroviral oncogene product, i.e., those peptide determinants that show sequence differences with the positionally homologous determinants of the cellular proto-oncogene product. The induction of this reactivity does not in itself have vaccine potential, since the foreign determinants specific to the retroviral oncogene product are normally absent

20   from the cellular proto-oncogene product. Nevertheless, the foreign peptide elements, notably those that differ by only a single amino acid from the positionally homologous self peptides, trigger peripheral T-lymphocytes exhibiting a weak cross-reactivity for the self peptides. Although such self peptides are present in normal cells expressing the proto-oncogene, targeting of

25   the tumor cells is favored in view of their overexpression of the proto-oncogene.

It is possible that many tumor-associated and overexpressed proto-oncogenes might possess mutations. In some cases, overexpression may very well arise as a direct consequence of one or more of the mutations. However, the present vaccination method does not have as its object the

30   deliberate targeting of non-self determinants generated by proto-oncogene mutations. Unlike prior vaccination methods designed to target such mutation-

driven non-self determinants, it is the aim of the present invention to induce reactivity for self-determinants in the overexpressed product of tumor associated and overexpressed proto-oncogenes.

Prior efforts attempting to elicit reactivity to proto-oncogene self determinants have relied on *in vitro* protocols utilizing mutant cell lines to identify individual self peptide immunogens (Disis *et al.*, *Cancer Res.* (1994) 54:1071-1076; Peoples *et al.*, *Proc. Natl. Acad. Sci USA* (1995), 92:432-436). According to the present invention, the host immune system is presented with the full array of naturally-derived class I binding peptides. The vaccine strategy of the present invention obviates the need for any *a priori* assessment of the immunogenicity of individual peptides.

While the cellular immunogens of the invention display self peptides, non-self peptides would also be presented which may serve as more effective tolerance breakers. The value of a non-self, but closely related to self, peptide is that it may more readily activate those T cells that have both a weak cross reactivity for the cognate self peptide and an activation threshold (determined by the tightness of binding to the T cell receptor) too high to be triggered by the self peptide. Moreover, cognate non-self is inductive of a good immune response, simply because it does in fact constitute nonself. The non-self immune response is expected to predispose the induction of the inevitably weaker response to the self determinants on the same protein product, since the resultant cytokine release provides local help to initiate the weaker anti-self response.

As hereinafter exemplified in a model of *src*-oncogene-based tumor formation, immunization with cells transfected with a transgene construct expressing the v-*src* oncogene product induces reactivity to the product of the c-*src* proto-oncogene, thereby conferring protection against the growth of tumors displaying overexpression of the c-*src* proto-oncogene.

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Target Proto-Oncogenes

According to the present invention, patients with a family history of a cancer characterized by the overexpression of a particular proto-oncogene are selected for immunization. Alternatively, patients whose tumors can be shown to overexpress the proto-oncogene are selected. Overexpression of a proto-oncogene may derive from an increase over a basal level of transcription. Overexpression may also derive from gene amplification, that is, an increase in gene copy number, coupled with a basal or elevated level of transcription. Proto-oncogene overexpression may be assayed by conventional probing techniques, such as described in *Molecular Cloning: A Laboratory Manual* J. Sambrook *et al.*, eds., Cold Spring Harbor Laboratory Press, 2nd ed. 1989. The level of target proto-oncogene expression may be determined by probing total cellular RNA from patient cells with a complementary probe for the relevant mRNA. Total RNA from the patient cells is fractionated in a glyoxal/agarose gel, transferred to nylon and hybridized to an appropriately labelled nucleic acid probe for the target mRNA. The number of relevant mRNA transcripts found in the patient cells is compared to that found in cells taken from the same tissue of a normal control subject.

As an alternative to measuring mRNA transcripts, the expression level of a target proto-oncogene may be assessed by assaying the amount of encoded protein which is formed. Western blotting is a standard protocol in routine use for the determination of protein levels. See *Molecular Cloning, supra*, Chapter 18, incorporated herein by reference. Accordingly, a cell lysate or other cell fraction containing protein is electrophoresed on a polyacrylamide gel, followed by protein transfer to nitrocellulose, and probing of the gel with an antibody specific for the protein in question. The probe step permits resolution of the desired protein from all other proteins in the starting mixture. The bound antibody may be prelabeled, *e.g.*, by a radioisotope such as ^{125}I, so as to permit its detection on the gel. Alternatively, a secondary reagent (usually an anti-immunoglobin or protein A) may be radiolabeled or covalently coupled

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to an enzyme such as horseradish peroxidase or alkaline phosphatase. The strength of the signal is proportional to the amount of the target protein. The strength of the signal is compared with the signal from a sample analyzed in the same manner, but taken from normal as opposed to tumor tissue.

A description of the methodology and use of Western blotting to determine the levels of the c-*src*-encoded protein pp60[c-src] in adenomatous polyps (colonic epithelia) is provided by Cartwright *et al.*, *Proc. Natl. Acad. Sci. USA* (1990), 87:558-562, the entire disclosure of which is incorporated herein by reference.

An at least about eight-fold increase in that gene's expression in the patient cells compared to expression in normal control cells from the same tissue would indicate candidacy for vaccination.

Table 1 includes a partial list of representative proto-oncogenes, the overexpression of which has been associated with one or more malignancies. Each listed proto-oncogene is a target proto-oncogene according to the present invention. The corresponding oncogene, of which the target proto-oncogene is the normal cellular homolog, is also identified. This list of target proto-oncogenes is intended to be representative, and not a complete list.

Table 1
Representative List of Target Proto-Oncogenes

Proto-Oncogene	Tumor	Comments/References
AKT-2	ovarian	v-*Akt* is the oncogene of the AKT8 virus, which induces lymphomas in mice. 1. Bellacosa *et al.*, (1995) *Int. J. Cancer* 64(4):280-5: Southern-blot analysis has shown AKT-2 amplification in 12.1% of ovarian carcinomas, while Northern bot analysis has

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revealed overexpression of AKT-2 in 3 of 25
fresh ovarian carcinomas which were negative for
AKT-2 amplification.

2. Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci.
USA* 89(19): 9267-71: Amplification of AKT-2
has been detected in 10% of pancreatic
carcinomas.

AKT-2	pancreatic	Cheng *et al.*, (1996) *Proc. Natl. Acad. Sci. USA* 93(8):3636-41: Amplification of AKT-2 has been detected in 10% of pancreatic carcinomas.

c-*erb*B-2   bladder   c-*Erb*B-2 is also known as HER2/*neu*. V-*erb*B is
the oncogene of the avian erythroblastosis virus.

1.   Underwood *et al.*, (1995) *Cancer Res.*
55(11):2422-30:   Protein overexpression · was
observed in 45% of patients with non-recurrent
disease and 50% of patients with recurrent
disease; 9% of bladder tumors analyzed shoed
gene amplification.

2.   Coombs *et al.*, (1993) *Pathology* 169(1):35-
42: c-*Erb*B-2 gene amplification was observed in
14% of bladder tumors analyzed.

3. Gardiner *et al.*, (1992) *Urolog. Res.* 20(2):17-
20: Nineteen percent of primary transitional cell
bladder carcinomas showed   c-*erb*B-2 gene
amplification.

c-*erb*B-2   breast   1.   Molina *et al.*, (1966) *Anticancer Research*
16(4B):2295-300: Abnormal c-*erb*B-2 levels were
found in 9.2% of patients with locoregional breast

carcinoma, and in 45.4% of patients with advanced disease. 2. DePotter *et al.*, (1995) *Virchows Arch.* 426(2):107-15: Overexpression of the oncoprotein is observed in about 20% of invasive duct cell carcinomas of the breast. 3. Bandyopadhyay *et al.*, (1994) *Acta Oncol.* 33(5):493-8: 35.4% of breast tumors showed c-*erb*B-2 overexpression; 17.4% showed gene amplification. 4. Fontana *et al.*, (1994) *Anticancer Res.* 14(5B):2099-104: 26% of samples showed c-*erb*B-2 amplification. 5. Press *et al.*, (1993) *Cancer Research* 53(20):4960-70: Amplified overexpression was identified in 38% of primary breast cancers. 6. Berns *et al.*, (1992) *Cancer Res.* 52(5):1107-13: 23% of primary breast cancer tissues exhibited amplification. 7. Delvenne *et al.*, (1992) *Eur. J. of Cancer* 28(2-3):700-5: c-*erb*B-2 mRNA was overexpressed in 34% of breast tumor samples. 8. Inglehart, (1990) *Cancer Res.* 50(20):6701-7: Two to thirty-two-fold gene amplification was found in multiple stages of tumor progression. 9. Slamon *et al.*, (1989) *Science* 244:707-12: A 28% incidence of amplification of c-*erb*B-2 was found in 189 primary breast cancers. 10. Kraus *et al.*, (1987) *EMBO J.* 6(3):605-10: Eight cell lines demonstrated c-*erb*B-2 mRNA levels ranging from 4 to 128-fold overexpression. 60% of all tumors analyzed showed elevated levels of c-*erb*B-2 mRNA.

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| c-*erb*B-2 | lung | 1. Osaki *et al.*, (1995) *Chest* 108(1):157-62: Lung tissue overexpression of c-*erb*B-2 was discovered in 42.5% of samples. 2. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 64-fold increase in the amount of c-*erb*B-2 mRNA was observed; 33% of lung tumors showed overexpression of c-*erb*B-2. |

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| c-*erb*B-2 | ovarian | 1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Abnormally high expression of c-*erb*B-2 was found in 31% of tumor samples. 2. Felip *et al.*, (1995) *Cancer* 75(8):2147-52: 21.7% of ovarian tumors showed overexpression of c-*erb*B-2. 3. Fan *et al.*, (1994) *Chin. Med. J.* 107(8):589-93: c-*erb*B-2 amplification was found in 30.8% (8 of 26) of human ovarian cancers. 4. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 24% of ovarian tumors showed c-*erb*B-2 overexpression. 5. Csokay *et al.*, (1993) *Eur. J. of Surg. Oncology* 19(6):593-9: c-*erb*B-2 amplification was found in 34% of fresh ovarian tumor samples. 6. McKenzie *et al.*, (1993) *Cancer* 71(12):3942-5: 30% of ovarian tumor samples indicated c-*erb*B-2 overexpression. 7. Hung *et al.*, (1992) *Cancer Letters* 61(2):95-103: A 100-fold c-*erb*B-2 overexpression was discovered in one human cell line. Two to four-fold amplification was also discovered. |

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| *MDM*-2 | leukemia | *MDM*-2 is the murine double minute-2 oncogene. 1. Bueso-Ramos *et al.*, (1993) *Blood* 82(9):2617- |

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23:    53% of cases showed overexpression of *MDM*-2 mRNA. The level of *MDM*-2 mRNA overexpression in some cases of leukemias was comparable to that observed in some sarcomas, which demonstrate more than 50-fold *MDM*-2 gene amplification.   No evidence of gene amplification was observed. 2. Watanabe *et al.*, (1994) *Blood* 84(9):3158-65:   28% of patients with B-cell chronic lymphocytic leukemia or non-Hodgkin's lymphoma had 10-fold higher levels of *MDM*-2 gene expression. *MDM*-2 overexpression was found more frequently in patients at advanced clinical stages.

c-*myb*   colon   V-*myb* is   the   oncogene   of   the   avian myeloblastoma virus. 1. Ramsay *et al.*, (1992) *Cell Growth and Diff.* 3(10):723-30: c-*myb* levels were always higher in colon cancer samples than normal tissue. 2. Alitalo *et al.*, (1984) *Proc. Natl. Acad. Sci.* 81(14):4534-8: c-*myb* levels were always higher in colon cancer samples than normal tissue.

c-*myc*   breast   V-*myc* is the oncogene of the avian myelocytoma virus.   1.   Lonn *et al.*, (1995) *Cancer* 75(11):2681-7: Amplification of c-*myb* occurs in 16% of patients with breast cancer. 2. Hehir *et al.*, (1993) *J. of Surg. Oncology* 54(4):207-9: c-*myc* overexpression was found in 60% of breast carcinoma samples. 3. Kreipe *et al.*, (1993) *Cancer Research* 53(8):1956-61: Amplification of

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c-*myc* was found in 52.6% of samples that displayed a Ki-S1 labelling index exceeding 30%. 4. Watson *et al.*, (1993) *J. Nat. Cancer Inst.* 85(11):902-7: Amplification of c-*myc* occurs in up to 20 - 30% of breast cancers. 5. Berns *et al.*, (1992) *Cancer Research* 52(5):1107-13: Amplification was found in 20% of primary breast cancer patients; the range was 3-14 gene copies. 6. Watanabe *et al.*, (1992) *Cancer Research* 52(19):5178-82: Expression of c-*myc* was increased by 10-fold.

| c-*myc* | gastric/colorectal | 1. Rigas, (1990) *Clin. Gastroent.* 12(5):494-9: Overexpression of c-*myc* is found in 80 of colon cancers. 2. Erisman *et al.*, (1988) *Oncogene* 2(4):367-78: Adenocarcinoma cell lines express 5-10-fold elevated levels of c-*myc* mRNA. Eight to thirty-seven-fold higher levels of c-*myc* protein was found in tumor cell lines compared to normal cells. 3. Sikora *et al.*, (1987) *Cancer* 59(7):1289-95: Up to 32-fold overexpression of c-*myc* mRNA was observed in 12 to 15 tumors. 4. Tsuboi *et al.*, (1987) *Biochem. and Biophys. Res. Comm.* 146(2):705-10: Gastric Cancer: A 2-3-fold overexpression was observed in gastric cancer. A 2-10-fold overexpression was observed in colorectal cancer. |

| c-*myc* | lung | 1. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: A 57-fold increase in c-myc mRNA levels was observed. 23% of samples indicated strong |

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expression of c-myc. 2. Kato *et al.*, (1993) *Jap. J. of Cancer Res.* 84(4):355-9: Liver tissue metastases from human small cell lung carcinoma revealed 30-fold amplification of c-*myc*.

| 5 | c-*myc* | naso-pharn-geal | Porter *et al.*, (1994) *Acta Oto-Laryng.* 114(1): 1105-9: 22% of samples showed intense staining for c-*myc*. |

c-*myc*   ovarian   1. Bian *et al.*, (1995) *Chin. J. of Ob. Gyn.* 30(7):406-9: 50% of samples showed

10   amplification of c-*myc*. 2. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: 26% of samples exhibited c-*myc* amplification. 3. van Dam *et al.*, (1994) *J. Clin. Path.* 47(10):914-9: Overexpression of c-*myc* was found in 35% of

15   ovarian carcinomas. 4. Xin *et al.*, (1993) *Chin. J. of Ob. Gyn.* 28(7):405-7: 54.5% of samples showed amplification of c-*myc*. 5. Tashiro *et al.*, (1992) *Int. J. of Cancer* 50(5):828-33: Overexpression was found in 63.5% of all serous

20   adenocarcinoma tissues and 37.3% of all ovarian carcinoma tissues. Significant overexpression of c-*myc* was observed at Stage III compared with other stages.

c-*myc*   prostate   Nag *et al.*, (1989) *Prostate* 15(2):115-22: A 10-

25   fold amplification of c-*myc* was observed. Fifty-fold higher levels of mRNA transcripts of c-*myc* were found.

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c-*ras*	lung	*Ras* oncogenes were first recognized as the transforming genes of Harvey and Kirsten murine sarcoma viruses. Lorenz *et al.*, (1994) *Clin. Invest.* 72(2):156-63: a 13-fold increase in overexpression of c-Ki-*ras* was observed. 18% of tumors displayed strong overexpression of c-Ki-*ras*.
c-*ras*	ovarian	1. Katsaros *et al.*, (1995) *Anticancer Res.* 15(4):1501-10: Higher levels of *ras* protein than in normal or benign ovarian tumors were found in 45% of tumor samples. 2. vanDam *et al.*, (1994) *J. of Clin. Path.* 47(10):914-9: 20% of ovarian tumors exhibited c-*ras* overexpression. The levels of expression of c-*ras* were much higher in tumors of patients with recurrent or persistent disease after chemotherapy, than in the tumors of patients at initial presentation.
c-*src*	breast	V-*src* is the oncogene of the Rous sarcoma virus, which induces sarcomas in chickens. Muthuswamy *et al.*, (1994) *Mol. and Cell. Biol* 14(1):735-43: c-*erb*B-2-induced mammary tumors possessed 6-8-fold higher c-*src* kinase activity than adjacent epithelium.
c-*src*	colon/ colorectal	1. Cartwright *et al.*, (1994) *J. of Clin. Invest.* 93(2):509-15: c-*src* activity is 6-10-fold higher in mildly dysplastic ulcerative colitis (a chromic inflammatory disease of the colon with a high on incidence of colon cancer) than in non-dysplastic

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epithelia. This data suggests that activation of c-*src* is an early event in the genesis of UC colon cancer. 2. Talamonti *et al.*, (1993) *J. of Clin. Invest.* 91(1):53-60: High level of c-*src* activity from colorectal cancer is found in liver metastases. 3. Termuhlen *et al.*, (1993) *J. of Surg. Res.* 54(4):293-8: Colon carcinoma metastases to the liver had significantly increased activity of c-*src* with an average 2.2-fold increase. Extrahepatic colorectal metastases demonstrated an average 12.7-fold increase in c-*src* activity over normal mucosa.

c-*yes*   colon

V-*yes* is the oncogene of two avian sarcoma viruses, Esh sarcoma virus and Y73. 1. Pena *et al.*, (1995) *Gastroent.* 108(1):117-24: Twelve to fourteen-fold higher expression of c-*yes* was found in colonic transforming oncogene adenomas compared to normal mucosa. Activity of c-*yes* was elevated in adenomas that are at greatest risk for developing cancer. 2. Park *et al.*, (1993) *Oncogene* 8(10):2627-35: A ten to 20-fold higher than normal activity of c-*yes* was observed in 3 out of 5 colon carcinoma cell lines. A 5-fold higher than normal activity was found in 10 out of 21 primary colon cancers, compared to normal colonic cells.

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Selection of Cognate Transgene for Preparation of Cellular Immunogen

According to the present invention, a transgene construct is engineered comprising a transgene which is cognate to the target proto-oncogene (hereinafter "cognate transgene" or "CTG"). The transgene is selected such that
5   it encodes a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene. The transgene should be expressed to very high levels in the transfectants. Thus, the construct should contain a strong promoter.

The product encoded by the cognate gene must have a high
10 degree of sequence homology with the product of the target proto-oncogene, but also must display some amino acid differences with the target proto-oncogene product. Thus, there must be a subset of one or more amino acid differences between the target proto-oncogene and its cognate in order to provide immunogenic stimulus. Two classes of genes that satisfy these criteria are
15 retroviral oncogenes and xenogenic proto-oncogenes. The word "xenogenic" is intended to have its normal biological meaning, that is, a property or characteristic referring or relating to a different species. Thus, a xenogenic proto-oncogene is meant to include the a homologous proto-oncogene of a species other than the host organism species. It may be appreciated that in the
20 case of a target proto-oncogene, e.g. MDM2, for which no retroviral homolog is yet known, a xenogenic homologue is advantageously utilized as the source of the DNA for the cognate transgene.

In principle, a more effective immunogenic stimulus would depend on the particular sequence, and not on the distinction between a
25 retroviral oncogene and a xenogenic proto-oncogene in terms of their relative transforming capacity. Thus, in certain cases, a retroviral oncogene may be better at providing a tolerance-breaking immunogenic stimulus, and in other cases, a xenogenic proto-oncogene may be more effective.

The retroviral oncogene or xenogenic proto-oncogene DNA
30 forming the CTG may comprise the wild type oncogene or proto-oncogene DNA. More preferably, a mutant DNA is utilized, which is engineered so as

to be non-transforming in the host. The DNA is mutated to include one or more nucleotide insertions, deletions or substitutions which will encode an oncogene product which is nontransforming in the host, but retains the requisite degree of sequence homology with respect to the target proto-oncogene. A

5   cognate transgene deletion mutant (hereinafter "dCTG") is preferred.

A protein sequence is generally considered "cognate" with respect to the target proto-oncogene-encoded protein if it is evolutionarily and functionally related between species. A more precise view of cognation is based upon the following sequence comparison carried out utilizing the FASTA

10   program of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* (1988), 85:2444-2448, the entire disclosure of which is incorporated herein by reference. Cognation is attained upon satisfying two criteria imposed by FASTA; (i) alignment of segments corresponding to at least 75% of the target proto-oncogene's encoded amino acid sequence; (ii) at least 80% amino acid identity

15   within the aligned sequences. The segments of the target proto-oncogene protein sequence and protein test sequence satisfying the two criteria are referred to as "homology regions". Accordingly, at least 75% of the target proto-oncogene protein sequence is alignable with the test sequence. The alignable segments or homology regions may, however, represent less than 75%

20   of the total test polypeptide chain for the case of test sequences that may significantly exceed the target proto-oncogene protein in length.

One skilled in the art, armed with the FASTA program, may survey existing sequence data bases (either protein sequences or DNA sequences, insofar as the amino acid sequence is determined by FASTA for all

25   reading frames) for test sequences which are cognate with respect to the target proto-oncogene. At the same time, one can isolate and then sequence what are very likely to be cognate test sequences (*e.g.* feline MDM-2, as likely to be cognate to human MDM-2) and use FASTA to verify the presumed cognation. according to the criteria set above. One may obtain the sequences of

30   presumptive cognate proto-oncogenes from a large number of mammalian

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sequences and screen these sequences with FASTA according to the aforesaid formulation of cognation.

Because the product encoded by a CTG differs at a small number of amino acid positions from the product encoded by the target proto-oncogene, an immunogenic stimulus is provided that (i) is directed against the foreign protein and (ii) with a lower probability, induce an anti-self response. The CTG is selected such that the gene product will yield the greatest immunogenic stimulus to induce anti-self reactivity. Provided that overall sequence homology (preferably greater than about 75%) is maintained, the presence of scattered amino acid differences is desired, since any one residue would likely have a relatively low probability of inducing self-reactivity. Moreover, the greatest number of residue differences would be advantageous, consistent with maintaining the requisite degree of general sequence homology.

The selection of amino acid modifications for the CTG may be facilitated by resort to available computer-based models used to identify immunogenic peptide fragments of polypeptides. These models could be employed to select CTGs which would possess the maximum number of immunogenic peptides for a given HLA haplotype.

Screening Procedure for CTG Selection

Notwithstanding the availability of computer-based algorithms which have some predictive value, it is desirable to design CTGs with resort to a screening procedure based on an actual experimental assay that can be HLA-haplotype specific. Accordingly, cells are biopsied from a normal volunteer of particular haplotype. The cells are transfected with a CTG construct, preferably a dCTG construct, satisfying the criteria set for cognition. More preferably, the cells are transfected with multiple dCTGs, preferably at least five dCTGs, satisfying the criteria for cognition. The at least five dCTGs are selected to display amino acid differences that essentially extend throughout the polypeptide chains of the encoded sequences. The transfected cells are then used to immunize the volunteer in accordance with the immunization method of the

present invention. After immunization, the human subject is tested in a standard delayed hypersensitivity (DH) reaction with 10^4-10^6 irradiated, autologous fibroblasts, as transfected with the same dCTG (or series of dCTGs) as used for the immunizing preparation. A positive DH reaction (induration) would verify

5   the induction of reactivity. The induction of reactivity in this assay is readily demonstrable because of the priming to the non-self determinants on the dCTG-encoded protein and the readout in the DH reaction of the same nonself determinants. Once DH reactivity is demonstrated in a DH reaction that directly tests the antigenicity of the non-self determinants encoded by the dCTG

10   (*i.e.*, priming with a non-self construct, DH testing with the same non-self construct), the subject can be then tested in a DH reaction based on testing with the autologous cells transfected with a dCTG derived from the human proto-oncogene itself (*i.e.*, priming with a non-self construct, testing with the human self construct). Testing of a battery of human volunteers will lead to a

15   catalogue of HLA-matched dCTGs, such that, for individuals of the same HLA haplotype, the use of the particular dCTG would be inductive of reactivity to proto-oncogene-encoded self. Different CTGs may thus be tested so as to correlate maximal secondary stimulation with a particular HLA haplotype.

At the same time, this procedure may be used with patients

20   undergoing tumor resection (if post-operative immuno-suppressive protocols are not mandatory), such that prior to resection, a course of immunization would have been initiated, the endpoint of which would represent the development of a DH reaction.

Any given amino acid difference between the CTG-encoded

25   product and the proto-oncogene-encoded product has a low probability of being a "tolerance-breaker". Thus, it is preferable to transfect the host cells with a mixture of multiple different CTGs, preferably dCTGs. The number of different dCTGs is preferably five or more. Moreover, it is preferred that, among themselves, the multiple dCTGs show amino acid differences that

30   essentially extend throughout the polypeptide chains of the encoded sequences. The dCTGs would be selected to maximize amino acid differences and, at the

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same time, make sure that differences are found all along the polypeptide chain. It would thus not be preferable to select a battery of deletions all from within the same domain of the polypeptide chain.

According to a protocol which utilizes 10^7 irradiated cells for immunization containing five separate dCTGs, five groups of 2 X 10^6 cells are included in one inoculate, each group of 2 X 10^6 having been transfected with a separate dCTG from the total set of five CTGs that are cognate to a particular proto-oncogene.

Selection of Non-Transforming Cognate Transgenes

Non-transforming cognate transgene variants are most advantageously derived via deletion of a sequence essential for transformation. Unlike point mutations which are potentially reversible due to back mutations, deletion mutations are irreversible. Furthermore, deletion mutations do not possess the inherent disadvantage attaching to point mutations, namely, even though the requirement for generation of an acceptable cognate transgene is for a qualitative difference with the wild type, i.e., non-transforming versus transforming, any given point mutation may be neutral or else quantitative in its effect, that is, the mutation may reduce but not totally eliminate transformability. Thus, according to a preferred embodiment of the invention, a deletion is created in a region of the cognate transgene which encodes an amino acid sequence required for transformation. Consonant with non-transformability, the smallest deletion possible so as to leave intact the bulk of the antigenicity of the transgene product is selected.

The engineering of a cognate transgene deletion mutant that satisfies these criteria is facilitated by reports of structure-function relationship in oncogene-encoded proteins. Such reports serve to identify regions of oncoproteins that are essential for transformation, as opposed to regions which are either neutral or serve merely to modulate transformability. Although such reports are usually based on *in vitro* transformation assays, and are therefore independent of immune effects, these studies can be exploited to aid in the

construction of non-transforming dCTGs for use in the practice of the present invention.

The deletion mutant is engineered to include at least a part of the region identified as critical for transformation. In those cases where essential amino acids have been identified, the deletion will span these residues. The engineering of any desired deletion can be readily accomplished by polymerase chain reaction (PCR) according to conventional PCR techniques, based upon the known nucleotide sequence of the unmutated cognate transgene.

The following describes a representative protocol for deriving a non-transforming dCTG of the smallest possible deletion, for use in the practice of the present invention. A test dCTG, engineered on the basis of known or ascertained transformation-specific domains, and driven by the strongest possible promoter, is used to transfect murine 3T3 cells. A sister culture of 3T3 cells is also transfected, with non-deleted CTG. Each CTG or dCTG cell culture is inoculated into nude mice, in the absence of any treatment to render the cells non-dividing. Those dCTGs which do not yield tumors in the mice even after prolonged observation are then utilized as transgenes for the biopsied human cells which, upon transfection with the transgene, will serve as a cellular vaccine according to the practice of the present invention. The dCTGs are selected with the smallest deletion mutant consonant with non-transformability.

Some CTGs representing xenogenic proto-oncogenes may not be tumorigenic in the 3T3/nude mouse assay. For any such non-transforming CTG, it is not essential to generate a dCTG. However, even given non-tumorigenicity in nude mice, it may be desirable to opt for generation of a deletion mutant when the transgene is based upon a xenogenic proto-oncogene. In such cases, the deletion would be engineered so as to remove the homologous region to that deleted in the particular dCTG that corresponds to the deletion in the corresponding retroviral oncogene dCTG.

Even though the transgene construct may comprise mutant oncogene or proto-oncogene DNA which is nontransforming, it is nevertheless preferable, as a safety measure, to treat the transfected cells to render them non-

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dividing before inoculation back into the host. The cells are irradiated with a radiation dosage sufficient to render them non-dividing.

Oncogenicity Assay of Cognate Transgenes

As a further safety measure, the oncogenicity of a given dCTG is preferably thoroughly tested prior to infection of the human host cells which are used as cellular immunogens according to the practice of the present invention. For example, an oncogenicity testing regimen may take the form of three separate assays: (i) dCTG transfection of NIH 3T3 cells, followed by inoculation into nude mice; (ii) dCTG transfection of human fibroblasts, followed by inoculation into nude mice; and (iii) dCTG transfection of human fibroblasts, followed by an *in vitro* test of anchorage-dependent growth. In principle, all three should be negative to validate the use of any given dCTG in the vaccination method of the present invention.

According to the oncogenicity assay (i), after stable transfection of NIH 3T3 cells with the test dCTG, the transfectants are inoculated into nude mice. Tumorigenicity of the transfectants in the mice is then evaluated according to standard protocols.

According to oncogenicity assay (ii), human fibroblasts are transfected with the test dCTG as proposed in the above human immunization protocol. After stable dCTG transfection of human fibroblasts, however, rather than carrying out X-irradiation of the transfectants to render them non-dividing, followed by inoculation of the irradiated transfectants back into the human host, the transfectants are directly inoculated into nude mice as a direct test of tumorigenicity. Given the greater susceptibility of murine 3T3 cells to oncogenic transformation, *vis a vis* primary human or murine transfectants fibroblasts, assay (ii) is probably much less sensitive than assay (i), but does have the advantage of offering a direct test of dCTG oncogenicity in human cells.

According to oncogenicity assay (iii), non-irradiated dCTG-transfected human fibroblasts are assayed for anchorage-dependent growth, *i.e.*

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colony formation in soft agar, as a test of dCTG transforming potential in human cells. Anchorage independence, as defined by the ability of cells to grow when suspended in semisolid medium, is a common phenotype acquired by human tumor cells, particularly those tumor cells of mesenchymal origin,

5   such as fibrosarcomas. While assay (iii) has no *in vivo* readout, it offers an independent test of the critical issue of dCTG oncogenicity in human cells.

The oncogenicity assays are performed according to published protocols. Assay (i), comprising dCTG transfection of NIH 3T3 cells followed by inoculation into nude mice, may be performed according to the protocol of

10   Stevens *et al.*, *Proc. Natl. Acad. Sci. USA* (1988), 85:3875-3879, including DNA transfection by the calcium phosphate coprecipitation method of Manohaven *et al.*, *Carcinogenesis* (1985), 6:1295-1301. Accordingly, NIH 3T3 cells (7.5 X 10^5 cells per 100-mm dish) are exposed to a calcium phosphate-DNA coprecipitate (40 μg of genomic DNA plus 3 μg of pSV2neo per dish) for

15   4 hours. Two days later, each dish is trypsinized and reseeded into a 175-cm^2 flask. For the next 10 days, cultures are selected in G418 (400 μg/ml), and the flasks are then trypsinized and cells are replated in the same flask to disperse the G418-resistant colonies into a diffuse lawn of cells. Two days later, the cells are harvested and washed with serum-free medium prior to injection. One

20   injection of 5 X 10^6 cells into the right flank and one injection of 1 X 10^7 cells into the left flank, each in a volume of 200 μl, are done on each nude mouse. Injection sites are monitored at 3- or 4-day intervals for 100 days. The sites are scored for the number of tumors induced per injection site.

Oncogenicity assay (ii), whereby dCTG transfection of human

25   fibroblasts followed by inoculation into nude mice, is carried out in the same manner as assay (i) except that for assay (ii) the human fibroblast transfectants are substituted for the murine 3T3 transfectants.

Assay (iii), involves a test of the *in vitro* anchorage-dependent growth of dCTG-transfected human fibroblasts. The assay is carried out as

30   described in Stevens *et al.*, *J. Cancer Res. and Clin. Oncol.* 1989, 115:118-128. 1 x 10^5 cells are seeded per 60-mm dish into 0.33% Noble agar over a

6-ml 0.5% agar base layer in Hams F10 supplemented with 6% fetal bovine serum. A portion of the agar suspension is diluted with Hams F10 plus 6% fetal calf serum to 200 cells/5 ml to determine the cloning efficiency of these cells when seeded into plastic 60-mm dishes. Agar dishes are fed with 1 ml

5   Hams F10 supplemented with 6% fetal bovine serum on the 1st and 15th day after seeding. Four weeks after seeding, all agar colonies >75 μm in diameter are counted and the colony counts are normalized to the plating efficiencies which aliquots of the initially seeded cells showed on plastic. This comparison, or normalization, of the agar colony counts to the plastic dish colony counts is

10   useful in identifying and correcting for any mechanical artifacts which might result from the seeding into agar of dead cells that had persisted from the initial transfection treatment or from heat-induced cell death, which might have occurred while suspending cells in molten agar during the process of seeding the agar dishes.

15   The following is a partial list of various deletions which, based upon published accounts of experiments with human or animal cells, are believed to render the identified CTG non-tumorigenic.

Table 2
Deletion Mutations Rendering Indicated Gene Non-Transforming

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
Akt-2 (c-akt) (mouse)	M95936; SEQ ID NO:3 (*Mus musculus* serine/threonine kinase)	480	148-234	Bellacosa *et al.*, *Science* (1991), 254:274-278; Bellacosa *et al.*, *Oncogene* (1993), 8(3):745-54.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-*neu* (c-erbB-2) (rat)	M11730; SEQ ID NO:4 (human tyrosine kinase-type receptor (HER2) gene	1255	1-731	Bargmann *et al.*, *EMBO* (1988), 7(7):2043-52; Bernards *et al.*, *Proc. Natl. Acad. Sci. USA* (1987), 84(19):6854-8.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
mdm-2 (human)	U33199; SEQ ID NO:5 (human mdm2-A mRNA); U33200; SEQ ID NO:6 (human mdm2-B mRNA); U33201; SEQ ID NO:7 (human mdm2-C mRNA); U33202; SEQ ID NO:8 (human mdm2-D mRNA); U33203;	489	9-155	Dubs-Poterszman, *Oncogene* (1995), 11(11):2445 -50.

CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
c-*myb* (human)	J02012; SEQ ID NO:10 (proviral oncogene v-*myb*)	640	275-327	Kalkbrenner *et al.*, *Oncogene* (1990), 5(5):657-61.
c-*myc* (human)	X00364; SEQ ID NO:11 (human c-*myc* oncogene)	439	129-144	Sarid *et al.*, *Proc. Natl. Acad. Sci. USA* (1987), 84(1):170-3.
v-*ras* (Harvey Murine Sarcoma Virus)	M77193; SEQ ID NO:12 (Rat sarcoma virus v-*ras* oncogene)	189	32-44	Zhang *et al.*, *Science* (1990), 249:162-5 (1990)

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CTG	Genbank accession number for sequence	Number of amino acids in gene	Amino acids deleted, rendering CTG non-transforming	References
v-src (Rous Sarcoma Virus)	U41728; SEQ ID NO:13 (RSV Schmidt-Ruppin A clone SRA-V; v-src gene)	526	430-433	Bryant et al., Mol. Cell. Bio. (1984), 4(5):862-6.
c-yes (chicken)	D00333; SEQ ID NO:14 (human c-yes-2 gene)	541	438-441	Zheng et al.; Oncogene (1989), 4(1):99-104.

<u>Engineering of Vectors for Host Cell Transfection</u>

The engineering of vectors for expression of a particular CTG, preferably a dCTG, is based on standard methods of recombinant DNA technology, *i.e.* insertion of the dCTG via the polylinker of standard or commercially available expression vectors. The dCTG is operably linked to a strong promoter. Generally speaking, a "strong" promoter is a promoter which achieves constitutively high expression of the dCTG in the transfected cells. Each promoter should include all of the signals necessary for initiating transcription of the relevant downstream sequence. These conditions are fulfilled, for example, by the pBK-CMV expression vector available from Stratagene Cloning Systems, La Jolla, CA (catalog no. 212209). The pBK-

CMV vector contains the cytomegalovirus (CMV) immediate early promoter. dCTGs xenogenic with respect to a particular target proto-oncogene may be isolated by conventional nucleic acid probing techniques, given the availability of a highly homologous probe represented by the cognate retroviral oncogene

5   and/or the human proto-oncogene itself.

Collection of Host Cells for Transfection

The host cells which may be transfected to derive the cellular immunogens of the present invention must express class I MHC and be susceptible to isolation and culture. Fibroblasts express class I MHC and may

10   be cultured. Accordingly, punch biopsies of host human skin are performed to harvest fibroblasts. Punch biopsies can be performed by a competent physician as a standard clinical procedure. Each biopsy yields a starting population of 1-2 X 10^7 cells that would proliferate in culture. Methods for the preparation of tissue cultures of human fibroblasts are well developed and widely used. *See*,

15   Cristofalo and Carpenter, *J. Tissue Culture Methods* (1980), 6:117-121, the entire disclosure of which is incorporated herein by reference. Essentially, skin obtained by punch biopsy is washed using an appropriate wash medium, finely minced and cultured in a suitable culture medium, such as Dulbecco's Modified Eagle Medium (DMEM), under CO_2 at 37°C. The cells are trypsinized with

20   a trypsin solution and transferred to a larger vessel and incubated at 37°C in culture fluid.

Host Cell Transfection

The expression vector carrying the dCTG is used to transfect biopsied host cells according to conventional transfection methods. One method

25   of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. *See* McCutchin and Pagano, *J. Natl. Cancer Inst.* (1968) 41:351-7. Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of Ca^{++} to a phosphate-containing DNA solution. The resulting precipitate

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apparently includes DNA in association with calcium phosphate crystals. These crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descen-

5   dants. *See* Graham *et al.*, *Virology* (1973), 52:456-467 and *Virology* (1974), 54:536-539.

Preferably, transfection is carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA)

10   or related liposome-forming materials. *See* Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* (1987) 84:7413-7417 (DNA-transfection); Malone *et al.*, *Proc. Natl. Acad. Sci. USA* (1989), 86:6077-6081) (RNA-transfection). One preferred technique utilizes the LipofectAMINE™ Reagent (Cat. No. 18324-012, Life Technologies, Inc., Gaithersburg, MD) which is a 3:1 (w/w) liposome

15   formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) (Chemical Abstracts Registry name: N-[2-({2,5-bis[(3-aminopropyl)amino]-1-oxypentyl}amino)ethyl]-N,N-dimethyl-2,3-bis(9-octadecenyloxy)-1-propanaminium trifluoroacetate), and the neutral lipid

20   dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water. Transfection utilizing the LipofectAMINE™ Reagent is carried out according to the manufacturer's published protocol. The protocol (for Cat. No. 18324-012) provides for either transient or stable transfection, as desired.

The advantage of transient expression is its rapidity, *i.e.* there is

25   no requirement for cellular proliferation to select for stable integration events. This rapidity could conceivably be of major clinical importance, in cases of an already metastatic tumor burden, wherein the weeks required for selection of stable transfectants may simply not be available to the clinician.

There are, nonetheless, two general disadvantages to the use of

30   transient transfection. The first is that expression usually peters out after a few days, in contrast to the continual expression in the case of stable transfection.

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This is not particularly crippling in terms of our immunization protocol. The inoculated, irradiated cells used for immunization would likely not survive *in vivo* for more than 4 or 5 days, in any case. Thus the nominal advantage accruing to stable transfection, that of a long-duration expression by the progeny

5   of the parental inoculated cell, is not of particular relevance in the case of the immunizing regime described herein, which is based on the use of non-dividing, probably short-lived cells.

A second disadvantage of transient transfection resides in the fact that it yields a cell population, only a subset of which has actually been

10   transfected and thus expresses the protein encoded by the transgene. This problem is obviated in the case of stable transfection, wherein over time one can develop a pure population of transfectants via selection for a resistance marker, such as *neo*, under conditions of clonal proliferation of the initial stable transfectants, *i.e.* daughter cells of transiently transfected cells lack the

15   transgene, in contrast to the case with stable transfectants. In the situation where there is sufficient time to effect immunization based on stably transfected cells, the progeny of all transfected clones would be utilized, not just the progeny of a single clone, as is sometimes done for detailed biochemical and molecular analyses of gene expression. Clearly the more clones utilized, the

20   more quickly one can arrive at the requisite number of cells to be used for immunization.

Percentage of Cells Exhibiting dCTG Expression

The percentage of cells exhibiting dCTG expression may be determined by an immunohistology assay. In this procedure, a small number

25   of cells (~ 500) from the harvested pellet following centrifugation of transfected cells are deposited on a cover slip and fixed with cold acetone. At this point, a standard immunohistological assay is carried out with the cells on the cover slip, *i.e.* addition of a primary monoclonal antibody reactive to the dCTG-encoded protein, followed by the addition of a developing antibody, *e.g.* a

30   fluorescent tagged antibody reactive to the primary monoclonal antibody.

Measurement of the percentage of cells scoring as dCTG-positive in the fluorescent assay allows a determination of the number of positive transfectants in the starting culture, and thus the number of total cells to be used for immunization to arrive at the desired number of dCTG-positive cells to be inoculated in the patient.

If, as would be almost certain, the percentage of cells scoring as dCTG-positive is less than one hundred percent, one can simply increase the number of cells to be used for immunization, so as to include the desired number of transfectants. The non-transfected cells in the immunizing population would simply represent x-irradiated, autologous fibroblasts that would constitute no danger to the patient.

Transfectant Irradiation

Prior to return to the host, the transfected cells are preferably irradiated. The transfectants are irradiated with a radiation dose sufficient to render them non-dividing, such as a dose of 25 By or 2500R. The cells are then counted by trypan blue exclusion, and about 2×10^7 irradiated transfectants are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

Vaccination Procedure

The transfected cells are returned to the host to achieve vaccination. The cells may be reimplanted at the same body site from which they were originally harvested, or may be restored to a different site.

It is the object of the present invention to generate a systemic tumor immune response, so as to fight metastasis formation wherever any metastases are found. Accordingly, there is no reason to inject the transfected cells at the same body site from which they were taken. Intramuscular or subcutaneous inoculation at a distal site would suffice to yield a systemic response. Thus, patients are preferably vaccinated by subcutaneous inoculation of the transfected cells.

For s-*crc* overexpression associated with colon carcinoma, partial venous inoculation is preferred, as the liver is a frequent site of metastases. For vaccinating against breast cancers and lymphomas, systemic immunization is preferred.

As a general rule, it is desirable to generate the strongest immune response consistent with clinical monitoring of no adverse side effects, *i.e.* multiple rounds of inoculation with, for example 10^7 cells, at each round. The number of rounds of inoculation is selected accordingly. The efficacy of the inoculation schedule may be monitored by a delayed hypersensitivity reaction administered to the patient. A course of about up to 10 inoculations, at 2-3 week intervals, may be utilized. It may be appreciated that the inoculation schedule may be modified in view of the immunologic response of the individual patient, as determined with resort to the delayed-type hypersensitivity (DTH) reaction.

Patient Response Monitoring by Delayed-type Hypersensitivity Reaction

Patients are assessed for reactivity to the irradiated transfectants by a test of skin reactivity in a DTH reaction. DTH has been used clinically (Chang *et al.* (1993), *Cancer Research* 53:1043-1050). To measure reactivity to the autologous irradiated transfectants, 10^4 - 10^6 cells in a volume of 0.1 ml Hanks buffered saline solution (HBSS) are inoculated intradermally into the host. Induration is measured 48 hours later, as an average of two perpendicular diameters (responses of greater than ≥ 2 mm is considered positive).

One advantage to the DTH assay is that it can independently assess the induction of T cell reactivity to (i) the transfectants used for immunization (*i.e.* the set of 5 or more dCTGs chosen for immunization purposes, each containing non-self determinants) and (ii) transfectants, as transfected with the human dCTG itself containing only self determinants. Thus, the induction of reactivity to the transfectants used for immunization establishes that the immunizing transfectants are in fact immunogenic, that is, the patient has not exhibiting a much weakened capacity for immune response.

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If the patient is demonstrably capable of response to the immunizing transfectants, then skin testing with the dCTG (human) transfectants would establish whether or not reactivity to the human proto-oncogene encoded product had been induced. According to the practice of the invention, inoculation of the
5   immunizing transfectants would continue for at least as long as the induction of reactivity to the human proto-oncogene-encoded protein occurs.

The practice of the invention is illustrated by the following nonlimiting examples.

Example 1

10   ## Immunization of Chickens Against c-*src*(527)-Induced Tumors By Vaccination with v-*src* DNA

A.   Genes

The oncogene c-*src*(527) is an activated form of chicken c-*src*. Its protein product pp60$^{c-src(527)}$ differs from the protein product of c-*src*, pp60^{c-}
15   ^{src}, by only a single amino acid substitution, phenylalanine for tyrosine at residue 527 (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73). This substitution eliminates the negative regulatory influence exerted on pp60^{c-src} phosphokinase activity by the enzymatic phosphorylation of the position 527 tyrosine. The protein product of v-*src*, pp60^{v-src}, shows a number of sequence differences with
20   pp60^{c-src} (Takeya and Hanafusa, (1983) *Cell* 32, 881-890), including scattered single amino acid substitutions within the first 514 residues and a novel C terminus of 12 amino acids (residues 515-526), in place of the nineteen C terminal amino acids of pp60^{c-src} (residues 515-533). Both the v-*src*-positive plasmid, pMv*src*, and the c-*src*(527)-positive plasmid, pc*src*527, were originally
25   shown (Kmiecik and Shalloway, (1987) *Cell* 49, 65-73) to transform murine NIH 3T3 cells in culture. However, the v-*src*-induced transformants exhibited a more rapid or more extensive colony growth in soft agarose than the c-*src*(527)-induced transformants, as well as a usually shorter latency of tumor formation in nude mice (*id.*).

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B.   Plasmids

1.   pvSRC-C1

The pVSRC-C1 plasmid was prepared as described by Halpern *et al.*, (1991) *Virology* 180, 857-86. Essentially, the plasmid was derived from the pRL^v-*src* plasmid (Halpern *et al.*, (1990) *Virology* 175, 328-331) by subcloning the v-*src*(+) *Xho*I-*Eco*RI fragment of the latter into the multiple cloning sequence of pSP65 (Melton *et al.*, (1984) *Nucleic Acids Res.* 12, 7035-7056) which had been cleaved with *Sal*I and *Eco*RI; since ligation of the *Xho*I overhang at the *Sal*I site destroys both recognition sequences, subsequent removal of the v-*src*(+) insert from the vector was achieved by digestion with *Eco*RI and with *Hind*III, which cleaves at a position in the multiple cloning sequence adjacent to the *Sal*I site. The pVSRC-C1 plasmid was restricted with *Eco*RI and *Hind*III, so as to liberate the tumorigenic insert. This insert included the v-*src* oncogene of the subgroup A strain of Prague RSV, as flanked downstream by a portion of the long terminal repeat (LTR) of RSV (from the 5' start of the LTR, to the single *Eco*RI site).

2.   pMv*src*

The pMv*src* plasmid was generously provided by Dr. David Shalloway, Cornell University, Ithaca, NY. The plasmid is prepared according to Johnson *et al.*, (1985) *Mol. Cell. Biol.* 5, 1073-1083. Briefly, the 3.1-kb *Bam*HI-*Bg*/II Schmidt Ruppin A v-*src* fragment from plasmid pN4 (Iba *et al.*, (1984) *Proc. Nat. Acad. Sci. USA* 81, 4424-4428) is inserted into the pEVX plasmid (Kriegler *et al.*, (1984) *Cell* 38,483-491) at a *Bg*/II site lying between two Moloney murine leukemia virus (MoMLV) long terminal repeats (LTRs). This fragment contains 276 bp of pBR322 DNA from the pBR322 *Bam*HI to *Sal*I sites followed by 2.8 kb of Rous sarcoma virus (RSV) DNA from the *Sal*I site that is about 750 bp upstream of the *env* termination codon down to the *Nru*I site that is about 90 bp downstream of the v-*src* termination codon. (The

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*Nru*I site is converted to a *Bg*/II site in the construction of pN4.) Ligation is performed by using a 10:1 insert-vector DNA fragment molar ratio.

The pMv*src* plasmid was restricted with NheI, so as to liberate a tumorigenic fragment. The fragment included the v-*src* oncogene of the subgroup A strain of Schmidt-Ruppin RSV, as flanked upstream by most of the Moloney murine leukemia virus (MoMLV) LTR (from the *Nhe*I site near the 5' start of the LTR, to the 3' end of this LTR) and downstream by a small portion of the MoMLV LTR (from the 5' start to the *Nhe*I site).

3.   p*csrc*527

The p*csrc*527 plasmid is prepared according to Kmiecik and Shalloway, (1987) *Cell* 49, 65-73. Briefly, a plasmid is constructed by cleaving expression vector pEVX (Kriegler *et al.*, (1984) *Cell* 38, 483-491 at its unique *Bg*III site lying between two MoMLV LTRs and inserting the 3.2 kilobase (kb) pair *Bam*HI-*Bg*III hybrid *src* fragment from plasmid pHB5 in the proper orientation. This fragment contains sequences from pBR322, the SRA *env* 3' region, SRA v-*src*, *src* from recovered ASV, and chicken c-*src*. The *Bg*III site is generated by insertion of a linker at the *Sac*I site about 20 bp downstream from the c-*src* termination codon. The restriction map of pMHB5 contains the MoMLV splice donor about 60 bp downstream from the 3'end of the upstream LTR and the v-*src* splice acceptor about 75 bp upstream from the *src* ATG.

Plasmid pMHB5527 is constructed by inserting the synthetic double-stranded DNA oligomer

```
5'        CCAGTTCCAGCCTGGAGAGAACCTATA (SEQ ID NO:1)          3'
3'   TCGGGGTCAAGGTCGGACCTCTCTTGGATATCTAG (SEQ ID NO:2)      5'
```

into pMHB5 between the *Ban*II site at c-*src* codon 524 and the downstream unique *Bg*III site. This alters the TAC Tyr 527 codon to a TTC Phe codon while preserving the remaining c-*src* coding region. Equimolar amounts of the double-stranded oligomer and three gel-purified tandem restriction fragments from pMHB5 are ligated in one reaction, which contains the following: the

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oligomer with *Ban*II and *Bg*III complementary ends, the 3 kb *Bg*III-*Bg*II (*Bg*II in the pEVX ampicillin resistance gene) partial digest fragment, the adjacent 6.1 kb *Bg*II-*Bg*II (downstream Bg*II* in c-*src*) fragment, and the 0.38 kb *Bg*II-*Ban*II (*Ban*II at c-*src* codon 524) fragment.

5   Plasmid pc*src*527 is constructed by replacing the 2 kb *Sal*I (in *env*)-*Mlu*I (in c-*src*) fragment in plasmid pMHB5527, with the homologous fragment from plasmid p5H. This fragment contains the coding sequence for the c-*src* amino region (codons 1 to 257) that have been isolated by molecular cloning of a c-*src* provirus and previously shown by sequencing to contain
10   authentic c-*src* sequence without the mutation at codon 63 (Levy *et al.*, (1986) *Proc. Natl. Acad. Sci. USA* 83, 4228-4232). Equimolar amounts of complementary gel-purified *Sal*I-*Mlu*I fragments from p5H and the other plasmids are ligated.

              The pc*src*527 plasmid was restricted with *Nhe*I, so as to liberate
15   a tumorigenic fragment. The tumorigenic fragment included the c–*src*(527) oncogene, as flanked by the same LTR complement as in pMv*src*.

C.   Animals

              Chickens of two closed lines, SC and TK, were utilized. These lines differ at the major histocompatibility (*B*) complex (B^2/B^2 for the SC line,
20   B^{15}/B^{21} for the TK line). Embryonated eggs were obtained from Hyline International (Dallas Center, IA). All chickens were hatched at the University of New Hampshire Poultry Research Farm and housed in isolation.

D.   Tumor Induction by Plasmid DNA

              Tumors were induced by subcutaneous inoculation in the wing
25   web of a *src*-positive plasmid according to the technique described by Fung *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80, 353-357 and Halpern *et al.*, (1990) *Virology* 175, 328-331. Of the three tumorigenic plasmids utilized here, all were adjusted, prior to inoculation, to a concentration of 100 µg of enzyme-- restricted DNA per 100 µl of phosphate-buffered saline. The conditions of

inoculation used for particular experiments (age of chicken at time of inoculation, amount of plasmid, etc.) are indicated below.

E.   Growth of Primary (wing web) Tumors in TK or SC Chickens Inoculated with pVSRC-C1, pMvsrc or pcsrc527

5   Individual 1-day-old chickens of line TK or of line SC were inoculated with 100 Ig of either pVSRC-C1, pMvsrc or pcsrc527. The mean tumor diameter (mm) at a particular time point and for any one group of TK or SC line chickens inoculated with an individual src-positive construct was computed as the sum of the diameters of the primary tumors divided by the

10   number of chickens surviving to that point. The results are shown in Fig. 1A (line TK) and Fig. 1B (line SC). The ratios at each time point show, for a particular group, the number of chickens bearing palpable tumors to the total number of survivors to that point (standard typeface for pcsrc527, italics for pVSRC-C1, bold typeface for pMVsrc). Error bars (unless obscured by the

15   symbol) indicate standard error.

F.   Growth of Challenge (wing web) Tumors in Test and Control Line TK Chickens Under Conditions of Priming and Homologous Challenge with pcsrc527, or Priming and Homologous Challenge with pVSRC-C1

20   Growth of challenge (wing web) tumors in test and control line TK chickens was determined under conditions of (i) priming and homologous challenge with pcsrc527, or (ii) priming and homologous challenge with pVSRC-C1. Test chickens were primed at 1 day posthatch with 100 Ig of construct; test and control chickens were challenged at five weeks posthatch

25   with 200 Ig of construct. The mean challenge tumor diameter was computed as described in the preceding section. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming and homologous challenge with pcsrc527 (Fig. 2A) and priming and homologous challenge with pVSRC-C1 (Fig. 2B)

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(standard typeface for control group, bold typeface for test group). The statistical comparison between the mean challenge tumor diameters of the test versus the control group at a particular time point was made using a two-tailed student's t test, *(p < 0.05), **(p < 0.01), ***(p < 0.001). The statistical comparison between the ratios of chickens bearing palpable challenge tumors to total number of survivors of the test versus the control group at a particular time point was made using a chi-squared test; the paired ratios are underlined for only those time points where p < 0.05. Error bars indicate standard error.

G.   Growth of Challenge (wing web) Tumors in Test and Control line TK chickens under Conditions of Priming with pVSRC-C1 and Heterologous Challenge with pcsrc527, or Priming with pcsrc527 and Heterologous Challenge with pVSRC-C1

Growth of challenge (wing web) tumors in test and control line TK chickens, was determined under conditions of (i) priming with pVSRC-C1 and heterologous challenge with pcsrc527, or (ii) priming with pcsrc527 and heterologous challenge with pVSRC-C1. Test chickens were primed at 1 day posthatch with 100 μg of construct; test and control chickens were challenged at five weeks posthatch with 200 μg of construct. The mean challenge tumor diameter was computed as described in Section E. At each time point the ratio of chickens bearing palpable challenge tumors to total number of survivors to that point is indicated for priming with pVSRC-C1 and heterologous challenge with pcsrc527 (Fig. 3A) and priming with pcsrc527 and heterologous challenge with pVSRC-C1 (Fig. 3B) (standard typeface for control group, bold typeface for test group). Statistical comparisons were made between test and control groups at a particular time point as described in the preceding section [*(p < 0.05), **(p < 0.01), ***(p < 0.001), for the student's t test], and the paired ratios are underlined for only those time points where, in the chi-squared test, p < 0.05. Error bars indicate standard error.

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H.   Discussion

In a direct comparison of the growth of tumors induced in line TK by either pMvsrc or pVSRC-C1, a similar pattern of relatively rapid regression was observed. This result established that the difference in LTR complement between these two v-src positive constructs did not exert a major influence on the tumor growth pattern in the TK line (Fig. 1A). By contrast, much more extensive and persistent tumor growth resulted from inoculation of TK chickens with the pcsrc527 construct (Fig. 1A). The relatively greater growth capacity of tumors induced by this construct indicated that in the TK line, the c-src(527) oncogene is much more highly tumorigenic than the v-src oncogene. This difference did not, however, generalize to the SC line (Fig. 1B). The SC line was chosen for comparison with the TK line on the basis of earlier observations (Halpern et al., (1993) Virology 197, 480-484) that v-src DNA-induced tumors engender a much weaker tumor immune response in line SC than in line TK. Whereas the growth of pcsrc527-induced primary tumors was virtually indistinguishable in the two lines, the growth of the v-src-induced tumors was considerably greater in the SC than in the TK line (Figs. 1A and 1B). Thus v-src, but not c-src(527), gives rise to primary tumors whose growth patterns differ in the two lines analyzed here.

Only minimal protection against homologous challenge was observed under conditions of priming to c-src(527) DNA, indicative of the induction of a relatively weak tumor immune response (Fig. 2A; a statistically significant lowering of challenge tumor growth in the test versus the control chickens was observed at only one time point). By contrast, the v-src DNA-primed chickens showed excellent protection against the homologous tumor challenge (Fig. 2B).

Priming with v-src DNA engenders a relatively greater degree of protection against challenge with c-src(527) DNA, than that afforded by priming with c-src(527) DNA itself (Fig. 3A). The degree of protection was weaker than that determined (Fig. 2B) for the case of priming and homologous challenge with v-src DNA. Only marginal protection was

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observed, however, when the heterologous challenge protocol was carried out in the reverse order (Fig. 3B). These results demonstrate that induction of reactivity to an antigenicity specified in tumor cells by an overexpressed proto-oncogene can confers tumor immunity.

5   **Example 2**

Vaccination Protocol

The following is a representative vaccination protocol according to the present invention.

A.   <u>Skin Punch Biopsy</u>

10   A punch biopsy of skin is obtained by a trained physician following standard medical practice.

B.   <u>Preparation of Primary Fibroblast Culture</u>

Under sterile conditions, the skin obtained by punch biopsy is put in a tube with 10 ml of the following wash medium: Dulbecco's Modified

15   Eagle Medium (DMEM), containing sodium bicarbonate (30 ml/liter of a 5.6% solution) and penicillin/streptomycin (2 ml/liter of a pen-strep stock solution containing 5000 units penicillin and 5000 μg of streptomycin/ml, pH 7.2-7.4.). In a sterile hood, the skin biopsy is added to a Petri dish, and then transferred several times to new Petri dishes containing the same wash medium. The

20   biopsy is then finely minced with two scalpels, and 2-4 pieces ($<$1 mm^3) of the minced biopsied are placed in the middle part of one or more T25 flasks. The flask is placed in a tissue culture incubator at 37°C for one half hour with the cap firmly closed, then opened for 10 minutes. The following culture medium is prepared: DMEM containing sodium bicarbonate; antibiotics; and 10% fetal

25   calf serum containing 2.5 μg/ml fungizone, 40 μg/ml gentamicin, and 1% glutamine(3% W/V). Two ml of the culture medium is then added to the flask, and the flask is incubated at 37°C (5% CO_2), with the cap lightly unscrewed. The flask is left for three days without moving so as to obtain adhesion of the

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separate pieces of skin to the plastic. Afterwards, the medium is changed two times per week over a 3-4 week period always adding 2-3 ml of medium. To trypsinize the skin cell culture, one needs zones of confluence. After aspirating the culture medium, 5 ml of the Puck's Saline A/EDTA solution (0.4 g EDTA

5   to 1 liter of Puck's Solution A) is added and immediately aspirated. Then 1 ml of trypsin solution (0.05/0.02% trypsin in PBS, without Ca++ or Mg++) is added and incubated for 5 min at 37°C, at which time 2 ml of culture fluid is added to stop the action of the trypsin. The cells are then transferred to a larger flask (T75) and incubated at 37°C in 15 ml of culture fluid, which is changed

10   every 2 days.

C.   Fibroblast Transfection

The fibroblasts (2 X 10^5 cells) are washed twice in DMEM without serum or antibiotics. A LipofectAMINE™-DNA solution is prepared by mixing in tube #1 mix 400μl DMEM and 10μl of dCTG vector DNA

15   (1μg/ul). In tube #2, 400 μl DMEM and 25 Ml of LipofectAMINE Reagent (Life Technologies, cat. no. 18324-012) are mixed. The contents of tube #1 and #2 are mixed together and are then left sitting at room temperature for 30 hours. Then, 3.2 ml of the LipofectAMINE™-DNA solution is added to the cells. The cells are incubated for six hours at 37°C, washed once with Hank's

20   Balanced Salt Solution, and then refed with growth medium and incubated for an additional 24 hours at 37°C

D.   Transfectant Irradiation

Transfectants are irradiated to a dose of 25 By or 2500R. the cells are then counted by trypan blue exclusion. 2 X 10^7 irradiated transfectants

25   are resuspended in a volume of 0.2-0.4 ml of Hanks Balanced Salt Solution.

E.   Vaccination

Patients are vaccinated by subcutaneous inoculation of 2 X 10^7 irradiated cells at 2-3 week intervals. A shorter or longer regimen is used,

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depending upon the results of delayed type hypersensitivity (DTH) reaction monitoring (described below).

F.   Patient Assessment by DTH Monitoring

            Patients are assessed for reactivity to the irradiated transfectants

5   by a test of skin reactivity in a DTH reaction, as described by Chang *et al.*
(1993), *Cancer Research* 53:1043-1050. To measure reactivity to the
autologous irradiated transfectants, 10^4 - 10^6 transfected irradiated cells in a
volume of 0.1 ml HBSS are inoculated intradermally. Induration is measured
48 hours later, as an average of two perpendicular diameters. Responses of

10   greater than 2 mm are considered positive.

Example 3
v-*myc* Transfection of Murine Fibroblasts

A.   Vector Preparation

            The v-*myc* retroviral oncogene of avian myelocytomatosis virus

15   MC29 (Land *et al.* (1983), *Nature* 304:596-602) was obtained from the
American Type Culture Collection, Rockville, MD, 20852, as the pSVv-*myc*
vector (ATCC No. 45014). The v-*myc*-positive *Eco*RI-*Kpn*I fragment of pSVv-
myc was ligated into the polylinker sites of the pBK-CMV plasmid (Stratagene
Cloning Systems, La Jolla, CA).

20   B.   Cell Transfection

            Stable transfection using the pBK-CMV-v-*myc* vector was carried
out on a line of A31 fibroblasts (Balb/c origin), obtained from the ATCC. 2
X 10^5 cells were seeded in a 100 mm/dish and allowed to grow for 18-20 h
(RPMI 1640 medium and 10% fetal bovine serum), at which time the cells

25   reached 50-70% confluence. The cells were then washed twice in Dulbecco's
Modified Eagles Medium (without serum or antibiotics). A LipofectAMINE™-
DNA solution was prepared according to Example 2.C., with the pBK-CMV-v-

myc vector DNA, and 3.2 ml of the LipofectAMINE™-DNA solution added to the cells. The cells were then incubated for 6 hours at 37°C, washed once with Hank's Balanced Salt Solution, and then refed with the growth medium and incubated for an additional 24 hour at 37°C. Thereafter, the cells were fed

5   once every two days with growth medium containing 250 μg/ml geneticin (G418; Gibco BRL cat. no. 11811) as the selective marker. Within two weeks, colonies were picked and expanded into permanent cell lines. The cells were then washed and collected by centrifugation.

It should be noted that the procedure for transient transfection is

10   the same, through the point of incubation with the Lipofectamine™-DNA solution. Thereafter, the cells are washed and incubated for 72 hours in growth medium.

All references cited with respect to synthetic, preparative and analytical procedures are incorporated herein by reference.

15   The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indication the scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Allegheny University of the Health Sciences
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               England, James M.

   (ii) TITLE OF INVENTION: CANCER VACCINE

  (iii) NUMBER OF SEQUENCES: 14

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    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

   (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

  (vii) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 60/010,262
        (B) FILING DATE: 19-JAN-1996

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 1599 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 4530 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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TTGTCCATTT GCAAATATAT TTTGGAAAAC                                    4530
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(2) INFORMATION FOR SEQ ID NO:5:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 891 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA      60

GCTTCGGAAC AAGAGACCCT GGATCTTGAT GCTGGTGTAA GTGAACATTC AGGTGATTGG     120

TTGGATCAGG ATTCAGTTTC AGATCAGTTT AGTGTAGAAT TTGAAGTTGA ATCTCTCGAC     180

TCAGAAGATT ATAGCCTTAG TGAAGAAGGA CAAGAACTCT CAGATGAAGA TGATGAGGTA     240

TATCAAGTTA CTGTGTATCA GGCAGGGGAG AGTGATACAG ATTCATTTGA AGAAGATCCT     300

GAAATTTCCT TAGCTGACTA TTGGAAATGC ACTTCATGCA ATGAAATGAA TCCCCCCCTT     360

CCATCACATT GCAACAGATG TTGGGCCCTT CGTGAGAATT GGCTTCCTGA AGATAAAGGG     420

AAAGATAAAG GGAAATCTC TGAGAAAGCC AAACTGGAAA ACTCAACACA AGCTGAAGAG     480
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GGCTTTGATG TTCCTGATTG TAAAAAAACT ATAGTGAATG ATTCCAGAGA GTCATGTGTT     540

GAGGAAAATG ATGATAAAAT TACACAAGCT TCACAATCAC AAGAAAGTGA AGACTATTCT     600

CAGCCATCAA CTTCTAGTAG CATTATTTAT AGCAGCCAAG AAGATGTGAA AGAGTTTGAA     660

AGGGAAGAAA CCCAAGACAA AGAAGAGAGT GTGGAATCTA GTTTGCCCCT TAATGCCATT     720

GAACCTTGTG TGATTTGTCA AGGTCGACCT AAAAATGGTT GCATTGTCCA TGGCAAAACA     780

GGACATCTTA TGGCCTGCTT TACATGTGCA AAGAAGCTAA AGAAAAGGAA TAAGCCCTGC     840

CCAGTATGTA GACAACCAAT TCAAATGATT GTGCTAACTT ATTTCCCCTA G             891
```

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 657 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA     60

GCTTCGGAAC AAGAGACCCT GGACTATTGG AAATGCACTT CATGCAATGA AATGAATCCC     120

CCCCTTCCAT CACATTGCAA CAGATGTTGG GCCCTTCGTG AGAATTGGCT TCCTGAAGAT     180

AAAGGGAAAG ATAAAGGGGA AATCTCTGAG AAAGCCAAAC TGGAAAACTC AACACAAGCT     240

GAAGAGGGCT TTGATGTTCC TGATTGTAAA AAAACTATAG TGAATGATTC CAGAGAGTCA     300

TGTGTTGAGG AAAATGATGA TAAAATTACA CAAGCTTCAC AATCACAAGA AAGTGAAGAC     360

TATTCTCAGC CATCAACTTC TAGTAGCATT ATTTATAGCA GCCAAGAAGA TGTGAAAGAG     420

TTTGAAAGGG AAGAAACCCA AGACAAAGAA GAGAGTGTGG AATCTAGTTT GCCCCTTAAT     480

GCCATTGAAC CTTGTGTGAT TTGTCAAGGT CGACCTAAAA ATGGTTGCAT TGTCCATGGC     540

AAAACAGGAC ATCTTATGGC CTGCTTTACA TGTGCAAAGA AGCTAAAGAA AAGGAATAAG     600

CCCTGCCCAG TATGTAGACA ACCAATTCAA ATGATTGTGC TAACTTATTT CCCCTAG       657
```

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 966 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA     60

GCTTCGGAAC AAGAGACCCT GGTTAGACCA AAGCCATTGC TTTTGAAGTT ATTAAAGTCT     120

GTTGGTGCAC AAAAAGACAC TTATACTATG AAAGAGGATC TTGATGCTGG TGTAAGTGAA     180

CATTCAGGTG ATTGGTTGGA TCAGGATTCA GTTTCAGATC AGTTTAGTGT AGAATTTGAA     240
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GTTGAATCTC TCGACTCAGA AGATTATAGC CTTAGTGAAG AAGGACAAGA ACTCTCAGAT   300

GAAGATGATG AGGTATATCA AGTTACTGTG TATCAGGCAG GGGAGAGTGA TACAGATTCA   360

TTTGAAGAAG ATCCTGAAAT TTCCTTAGCT GACTATTGGA AATGCACTTC ATGCAATGAA   420

ATGAATCCCC CCCTTCCATC ACATTGCAAC AGATGTTGGG CCCTTCGTGA GAATTGGCTT   480

CCTGAAGATA AAGGGAAAGA TAAAGGGGAA ATCTCTGAGA AAGCCAAACT GGAAAACTCA   540

ACACAAGCTG AAGAGGGCTT TGATGTTCCT GATTGTAAAA AAACTATAGT GAATGATTCC   600

AGAGAGTCAT GTGTTGAGGA AAATGATGAT AAAATTACAC AAGCTTCACA ATCACAAGAA   660

AGTGAAGACT ATTCTCAGCC ATCAACTTCT AGTAGCATTA TTTATAGCAG CCAAGAAGAT   720

GTGAAAGAGT TTGAAAGGGA AGAAACCCAA GACAAGAAG AGAGTGTGGA ATCTAGTTTG   780

CCCCTTAATG CCATTGAACC TTGTGTGATT TGTCAAGGTC GACCTAAAAA TGGTTGCATT   840

GTCCATGGCA AAACAGGACA TCTTATGGCC TGCTTTACAT GTGCAAGAA GCTAAAGAAA   900

AGGAATAAGC CCTGCCCAGT ATGTAGACAA CCAATTCAAA TGATTGTGCT AACTTATTTC   960

CCCTAG   966

(2) INFORMATION FOR SEQ ID NO:8:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 399 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA   60

GCTTCGGAAC AAGAGACCCT GGTTAGACAA GAAAGTGAAG ACTATTCTCA GCCATCAACT   120

TCTAGTAGCA TTATTTATAG CAGCCAAGAA GATGTGAAAG AGTTTGAAAG GAAGAAACC   180

CAAGACAAAG AAGAGAGTGT GGAATCTAGT TTGCCCCTTA ATGCCATTGA ACCTTGTGTG   240

ATTTGTCAAG GTCGACCTAA AAATGGTTGC ATTGTCCATG GCAAAACAGG ACATCTTATG   300

GCCTGCTTTA CATGTGCAAA GAAGCTAAAG AAAAGGAATA AGCCCTGCCC AGTATGTAGA   360

CAACCAATTC AAATGATTGT GCTAACTTAT TTCCCCTAG   399

(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 309 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGTGCAATA CCAACATGTC TGTACCTACT GATGGTGCTG TAACCACCTC ACAGATTCCA   60

GCTTCGGAAC AAGAGACCCT GGTTAGACCA AAGCCATTGC TTTTGAAGTT ATTAAAGTCT   120

```
GTTGGTGCAC AAAAAGACAC TTATACTATG AAAGAGGTTC TTTTTTATCT TGGCCAGTAT      180

ATTATGACTA AACGATTATA TGATGAGAAG CAACAACATA TTGTAAATGA TTGTGCTAAC      240

TTATTTCCCC TAGTTGACCT GTCTATAAGA GAATTATATA TTTCTAACTA TATAACCCTA      300

GGAATTTAG                                                              309
```

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1897 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
CACAGATAAG GTTATTTGGG TACCCTCTCG AAAAGTTAAA CCGGACATCG CCCAAAAGGA       60

TGAGGTGACT AAGAAAGATG AGGCGAGCCC TCTTTTTGCA GGCTGGAGGC ACATAGATAA      120

GAGAATTATC ACTCTACATT CATCTTTCTC AAAGATTAAT CTACTTGTGT GTTTTATATT      180

TCATTAGAAT CGGACAGATG TTCAGTGCCA GCACCGGTGG CAGAAAGTAT TAAACCCAGA      240

ACTTAACAAA GGTCCATGGA CTAAAGAGGA GGATCAAAGG GTAATAGAAC ACGTGCAGAA      300

ATACGGTCCA AAGCGCTGGT CGGACATTGC TAAGCATTTG AAGGGAAGGA TTGGAAAACA      360

GTGCAGGGAG AGGTGGCACA ACCATCTGAA TCCAGAAGTG AAGAAAACCT CCTGGACAGA      420

AGAGGAAGAT AGAATTATTT ACCAGGCACA CAAGAGACTG GGAAACAGAT GGGCAGAAAT      480

TGCAAAGTTG CTGCCTGGAC GGACTGATAA CGCTGTCAAG AACCACTGGA ATTCCACCAT      540

GCGCCGGAAG GTCGAGCAGG AGGGTTACCC GCAGGAGTCC TCCAAAGCCG GCCCGCCCTC      600

GGCAACCACC GGCTTCCAGA AGAGCAGCCA TCTGATGGCC TTTGCCCACA ACCCACCTGC      660

AGGCCCGCTC CCGGGGGCCG GCCAGGCCCC TCTGGGCAGT GACTACCCCT ACTACCACAT      720

TGCTGAGCCA CAAAATGTCC CTGGTCAGAT CCCATATCCA GTAGCACTGC ATATAAATAT      780

TATCAATGTT CCTCAGCCAG CTGCTGCAGC TATTCAGAGA CACTATACTG ATGAAGACCC      840

TGAGAAAGAA AAACGAATAA AGGAATTAGA GTTGCTACTT ATGTCGACTG AGAATGAACT      900

GAAAGGGCAG CAGGCATTAC CAACACAGAA CCACACAGCA AACTACCCCG GCTGGCACAG      960

CACCACGGTT GCTGACAATA CCAGGACCAG TGGTGACAAT GCGCCTGTTT CCTGTTTGGG     1020

GGAACATCAC CACTGTACTC CATCTCCACC AGTGGATCAT GGTTGCTTAC CTGAGGAAAG     1080

TGCGTCCCCC GCACGGTGCA TGATTGTTCA CCAGAGCAAC ATCCTGGATA ATGTTAAGAA     1140

TCTCTTAGAA TTTGCAGAAA CACTCCAGTT AATAGACTCC TTCTTAAACA CATCGTCCAA     1200

TCACGAGAAT CTGAACCTGG ACAACCCTGC ACTAACCTCC ACGCCAGTGT GTGGCCACAA     1260

GATGTCTGTT ACCACCCCAT TCCACAAGGA CCAGACTTTC ACTGAATACA GGAAGATGCA     1320

CGGCGGAGCA GTCTAGAGCT CAATTATAAT AATCTTGCGA ATCGGGCTGT AACGGGGCAA     1380
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GGCTTGACCG AGGGGACTAT AACATGTATA GGCGAAAAGC GGGGTCTCGG TTGTAACGCG   1440

CTTAGGAAGT CCCCTCGAGG TATGGCAGAT ATGCTTTTGC ATAGGGAGGG GGAAATGTAG   1500

TCTTAATCGT AGGTTAACAT GTATATTACC AAATAAGGGA ATCGCCTGAT GCACCAAATA   1560

AGGTATTATA TGATCCCATT GGTGGTGAAG GAGCGACCTG AGGGCATATG GGCGTTAACA   1620

GAACTGTCTG TCCTTGCGTC ATTCCTCATC GGATCATGTA CGCGGCAGAG TATGATTGGA   1680

TAACAGGATG GCACCATTCA TCGTGGCGCA TGCTGATTGG TGCGACTAAG GAGTTGTGTA   1740

ACCCACGAAT GTACTTAAGC TTGTAGTTGC TAACAATAAA GTGCCATTCT ACCTCTCACC   1800

ACATTGGTGT GCACCTGGGT TGATGGCCGG ACCGTCGATT CCCTGACGAC TGCGAACACC   1860

TGAATGAAGC TGAAGGCTTC AGGTACCCTT ACTTGAT   1897

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8082 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTTGTTTG GCCGTTTTAG GGTTGTTGG AATTTTTTTT TCGTCTATGT ACTTGTGAAT   60

TATTTCACGT TTGCCATTAC CGGTTCTCCA TAGGGTGATG TTCATTAGCA GTGGTGATAG   120

GTTAATTTTC ACCATCTCTT ATGCGGTTGA ATAGTCACCT CTGAACCACT TTTTCCTCCA   180

GTAACTCCTC TTTCTTCGGA CCTTCTGCAG CCAACCTGAA AGAATAACAA GGAGGTGGCT   240

GGAAACTTGT TTTAAGGAAC CGCCTGTCCT TCCCCCGCTG GAAACCTTGC ACCTCGGACG   300

CTCCTGCTCC TGCCCCCACC TGACCCCCGC CCTCGTTGAC ATCCAGGCGC GATGATCTCT   360

GCTGCCAGTA GAGGGCACAC TTACTTTACT TTCGCAAACC TGAACGCGGG TGCTGCCCAG   420

AGAGGGGGCG GAGGGAAAGA CGCTTTGCAG CAAAATCCAG CATAGCGATT GGTTGCTCCC   480

CGCGTTTGCG GCAAAGGCCT GGAGGCAGGA GTAATTTGCA ATCCTTAAAG CTGAATTGTG   540

CAGTGCATCG GATTTGGAAG CTACTATATT CACTTAACAC TTGAACGCTG AGCTGCAAAC   600

TCAACGGGTA ATAACCCATC TTGAACAGCG TACATGCTAT ACACACACCC CTTTCCCCCG   660

AATTGTTTTC TCTTTTGGAG GTGGTGGAGG GAGAGAAAAG TTTACTTAAA ATGCCTTTGG   720

GTGAGGGACC AAGGATGAGA AGAATGTTTT TTGTTTTTCA TGCCGTGGAA TAACACAAAA   780

TAAAAAATCC CGAGGGAATA TACATTATAT ATTAAATATA GATCATTTCA GGGAGCAAAC   840

AAATCATGTG TGGGGCTGGG CAACTAGCTG AGTCGAAGCG TAAATAAAAT GTGAATACAC   900

GTTTGCGGGT TACATACAGT GCACTTTCAC TAGTATTCAG AAAAAATTGT GAGTCAGTGA   960

ACTAGGAAAT TAATGCCTGG AAGGCAGCCA AATTTTAATT AGCTCAAGAC TCCCCCCCCC   1020

CCCCAAAAAA AGGCACGGAA GTAATACTCC TCTCCTCTTC TTTGATCAGA ATCGATGCAT   1080

TTTTTGTGCA TGACCGCATT TCCAATAATA AAAGGGGAAA GAGGACCTGG AAAGGAATTA   1140

AACGTCCGGT TTGTCCGGGG AGGAAAGAGT TAACGGTTTT TTTCACAAGG GTCTCTGCTG   1200

ACTCCCCCGG CTCGGTCCAC AAGCTCTCCA CTTGCCCCTT TTAGGAAGTC CGGTCCCGCG   1260

GTTCGGGTAC CCCCTGCCCC TCCCATATTC TCCCGTCTAG CACCTTTGAT TTCTCCCAAA   1320

CCCGGCAGCC CGAGACTGTT GCAAACCGGC GCCACAGGGC GCAAAGGGGA TTTGTCTCTT   1380

CTGAAACCTG GCTGAGAAAT TGGGAACTCC GTGTGGGAGG CGTGGGGGTG GGACGGTGGG   1440

GTACAGACTG GCAGAGAGCA GGCAACCTCC CTCTCGCCCT AGCCCAGCTC TGGAACAGGC   1500

AGACACATCT CAGGGCTAAA CAGACGCCTC CCGCACGGGG CCCCACGGAA GCCTGAGCAG   1560

GCGGGGCAGG AGGGGCGGTA TCTGCTGCTT TGGCAGCAAA TTGGGGGACT CAGTCTGGGT   1620

GGAAGGTATC CAATCCAGAT AGCTGTGCAT ACATAATGCA TAATACATGA CTCCCCCCAA   1680

CAAATGCAAT GGGAGTTTAT TCATAACGCG CTCTCCAAGT ATACGTGGCA ATGCGTTGCT   1740

GGGTTATTTT AATCATTCTA GGCATCGTTT TCCTCCTTAT GCCTCTATCA TTCCTCCCTA   1800

TCTACACTAA CATCCCACGC TCTGAACGCG CGCCCATTAA TACCCTTCTT TCCTCCACTC   1860

TCCCTGGGAC TCTTGATCAA AGCGCGGCCC TTTCCCCAGC CTTAGCGAGG CGCCCTGCAG   1920

CCTGGTACGC GCGTGGCGTG GCGGTGGGCG CGCAGTGCGT TCTCTGTGTG GAGGGCAGCT   1980

GTTCCGCCTG CGATGATTTA TACTCACAGG ACAAGGATGC GGTTTGTCAA ACAGTACTGC   2040

TACGGAGGAG CAGCAGAGAA AGGGAGAGGG TTTGAGAGGG AGCAAAAGAA AATGGTAGGC   2100

GCGCGTAGTT AATTCATGCG GCTCTCTTAC TCTGTTTACA TCCTAGAGCT AGAGTGCTCG   2160

GCTGCCCGGC TGAGTCTCCT CCCCACCTTC CCCACCCTCC CCACCCTCCC CATAAGCGCC   2220

CCTCCCGGGT TCCCAAAGCA GAGGGCGTGG GGGAAAAGAA AAAAGATCCT CTCTCGCTAA   2280

TCTCCGCCCA CCGGCCCTTT ATAATGCGAG GGTCTGGACG GCTGAGGACC CCCGAGCTGT   2340

GCTGCTCGCG GCCGCCACCG CCGGGCCCCG GCCGTCCCTG GCTCCCCTCC TGCCTCGAGA   2400

AGGGCAGGGC TTCTCAGAGG CTTGGCGGGA AAAAGAACGG AGGGAGGGAT CGCGCTGAGT   2460

ATAAAAGCCG GTTTTCGGGG CTTTATCTAA CTCGCTGTAG TAATTCCAGC GAGAGGCAGA   2520

GGGAGCGAGC GGGCGGCCGG CTAGGGTGGA AGAGCCGGGC GAGCAGAGCT GCGCTGCGGG   2580

CGTCCTGGGA AGGGAGATCC GGAGCGAATA GGGGGCTTCG CCTCTGGCCC AGCCCTCCCG   2640

CTGATCCCCC AGCCAGCGGT CCGCAACCCT TGCCGCATCC ACGAAACTTT GCCCATAGCA   2700

GCGGGCGGGC ACTTTGCACT GGAACTTACA ACACCCGAGC AAGGACGCGA CTCTCCCGAC   2760

GCGGGGAGGC TATTCTGCCC ATTTGGGGAC ACTTCCCCGC CGCTGCCAGG ACCCGCTTCT   2820

CTGAAAGGCT CTCCTTGCAG CTGCTTAGAC GCTGGATTTT TTTCGGGTAG TGGAAAACCA   2880

GGTAAGCACC GAAGTCCACT TGCCTTTTAA TTTATTTTTT TATCACTTTA ATGCTGAGAT   2940

GAGTCGAATG CCTAAATAGG GTGTCTTTTC TCCCATTCCT GCGCTATTGA CACTTTTCTC   3000

AGAGTAGTTA TGGTAACTGG GGCTGGGGTG GGGGGTAATC CAGAACTGGA TCGGGGTAAA   3060

GTGACTTGTC AAGATGGGAG AGGAGAAGGC AGAGGGAAAA CGGGAATGGT TTTTAAGACT   3120

ACCCTTTCGA GATTTCTGCC TTATGAATAT ATTCACGCTG ACTCCCGGCC GGTCGGACAT   3180

TCCTGCTTTA TTGTGTTAAT TGCTCTCTGG GTTTTGGGGG GCTGGGGGTT GCTTTGCGGT   3240

GGGCAGAAAG CCCCTTGCAT CCTGAGCTCC TTGGAGTAGG GACCGCATAT CGCCTGTGTG   3300

AGCCAGATCG CTCCGCAGCC GCTGACTTGT CCCCGTCTCC GGGAGGGCAT TTAAATTTCG   3360

GCTCACCGCA TTTCTGACAG CCGGAGACGG ACACTGCGGC GCGTCCCGCC CGCCTGTCCC   3420

CGCGGCGATT CCAACCCGCC CTGATCCTTT TAAGAAGTTG GCATTTGGCT TTTTAAAAAG   3480

CAATAATACA ATTTAAAACC TGGGTCTCTA GAGGTGTTAG GACGTGGTGT TGGGTAGGCG   3540

CAGGCAGGGG AAAAGGGAGG CGAGGATGTG TCCGATTCTC CTGGAATCGT TGACTTGGAA   3600

AAACCAGGGC GAATCTCCGC ACCCAGCCCT GACTCCCCTG CCGCGGCCGC CCTCGGGTGT   3660

CCTCGCGCCC GAGATGCGGA GGAACTGCGA GGAGCGGGGC TCTGGGCGGT TCCAGAACAG   3720

CTGCTACCCT TGGTGGGGTG GCTCCGGGGG AGGTATCGCA GCGGGGTCTC TGGCGCAGTT   3780

GCATCTCCGT ATTGAGTGCG AAGGGAGGTG CCCCTATTAT TATTTGACAC CCCCCTTGTA   3840

TTTATGGAGG GGTGTTAAAG CCCGCGGCTG AGCTCGCCAC TCCAGCCGGC GAGAGAAȦ   3900

AGAAAAGCTG GCAAAAGGAG TGTTGGACGG GGGCGGTACT GGGGGTGGGG ACGGGGGCGG   3960

TGGAGAGGGA AGGTTGGGAG GGGCTGCGGT GCCGGCGGGG GTAGGACAGC GGCTAGGGCG   4020

CGAGTGGGAA CAGCCGCAGC GGAGGGGCCC CGGCGCGGAG CGGGGTTCAC GCAGCCGCTA   4080

GCGCCCAGGC GCCTCTCGCC TTCTCCTTCA GGTGGCGCAA AACTTTGTGC CTTGGATTTT   4140

GGCAAATTGT TTTCCTCACC GCCACCTCCC GCGGCTTCTT AAGGGCGCCA GGGCCGATTT   4200

CGATTCCTCT GCCGCTGCGG GGCCGACTCC CGGGCTTTGC GCTCCGGGCT CCCGGGGGAG   4260

CGGGGGCTCG GCGGGCACCA AGCCGCTGGT TCACTAAGTG CGTCTCCGAG ATAGCAGGGG   4320

ACTGTCCAAA GGGGGTGAAA GGGTGCTCCC TTTATTCCCC CACCAAGACC ACCCAGCCGC   4380

TTTAGGGGAT AGCTCTGCAA GGGGAGAGGT TCGGGACTGT GGCGCGCACT GCGCGCTGCG   4440

CCAGGTTTCC GCACCAAGAC CCCTTTAACT CAAGACTGCC TCCCGCTTTG TGTGCCCCGC   4500

TCCAGCAGCC TCCCGCGACG ATGCCCCTCA ACGTTAGCTT CACCAACAGG AACTATGACC   4560

TCGACTACGA CTCGGTGCAG CCGTATTTCT ACTGCGACGA GGAGGAGAAC TTCTACCAGC   4620

AGCAGCAGCA GAGCGAGCTG CAGCCCCCGG CGCCCAGCGA GGATATCTGG AAGAAATTCG   4680

AGCTGCTGCC CACCCCGCCC CTGTCCCCTA GCCGCCGCTC CGGGCTCTGC TCGCCCTCCT   4740

ACGTTGCGGT CACACCCTTC TCCCTTCGGG GAGACAACGA CGGCGGTGGC GGGAGCTTCT   4800

CCACGGCCGA CCAGCTGGAG ATGGTGACCG AGCTGCTGGG AGGAGACATG GTGAACCAGA   4860

GTTTCATCTG CGACCCGGAC GACGAGACCT TCATCAAAAA CATCATCATC CAGGACTGTA   4920

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TGTGGAGCGG CTTCTCGGCC GCCGCCAAGC TCGTCTCAGA GAAGCTGGCC TCCTACCAGG    4980

CTGCGCGCAA AGACAGCGGC AGCCCGAACC CCGCCCGCGG CCACAGCGTC TGCTCCACCT    5040

CCAGCTTGTA CCTGCAGGAT CTGAGCGCCG CCGCCTCAGA GTGCATCGAC CCCTCGGTGG    5100

TCTTCCCCTA CCCTCTCAAC GACAGCAGCT CGCCCAAGTC CTGCGCCTCG CAAGACTCCA    5160

GCGCCTTCTC TCCGTCCTCG GATTCTCTGC TCTCCTCGAC GGAGTCCTCC CCGCAGGGCA    5220

GCCCCGAGCC CCTGGTGCTC CATGAGGAGA CACCGCCCAC CACCAGCAGC GACTCTGGTA    5280

AGCGAAGCCC GCCCAGGCCT GTCAAAAGTG GGCGGCTGGA TACCTTTCCC ATTTTCATTG    5340

GCAGCTTATT TAACGGGCCA CTCTTATTAG GAAGGAGAGA TAGCAGATCT GGAGAGATTT    5400

GGGAGCTCAT CACCTCTGAA ACCTTGGGCT TTAGCGTTTC CTCCCATCCC TTCCCCTTAG    5460

ACTGCCCATG TTTGCAGCCC CCCTCCCCGT TTGTCTCCCA CCCCTCAGGA ATTTCATTTA    5520

GGTTTTTAAA CCTTCTGGCT TATCTTACAA CTCAATCCAC TTCTTCTTAC CTCCCGTTAA    5580

CATTTTAATT GCCCTGGGGC GGGGTGGCAG GGAGTGTATG AATGAGGATA AGAGAGGATT    5640

GATCTCTGAG AGTGAATGAA TTGCTTCCCT CTTAACTTCC GAGAAGTGGT GGGATTTAAT    5700

GAACTATCTA CAAAAATGAG GGGCTGTGTT TAGAGGCTAG GCAGGGCCTG CCTGAGTGCG    5760

GGAGCCAGTG AACTGCCTCA AGAGTGGGTG GGCTGAGGAG CTGGGATCTT CTCAGCCTAT    5820

TTTGAACACT GAAAAGCAAA TCCTTGCCAA AGTTGGACTT TTTTTTTTCT TTTATTCCTT    5880

CCCCCGCCCT CTTGGACTTT TGGCAAAACT GCAATTTTTT TTTTTTATT TTTCATTTCC    5940

AGTAAAATAG GGAGTTGCTA AAGTCATACC AAGCAATTTG CAGCTATCAT TTGCAACACC    6000

TGAAGTGTTC TTGGTAAAGT CCCTCAAAAA TAGGAGGTGC TTGGGAATGT GCTTTGCTTT    6060

GGGTGTGTCC AAAGCCTCAT TAAGTCTTAG GTAAGAATTG GCATCAATGT CCTATCCTGG    6120

GAAGTTGCAC TTTTCTTGTC CATGCCATAA CCCAGCTGTC TTTCCCTTTA TGAGACTCTT    6180

ACCTTCATGG TGAGAGGAGT AAGGGTGGCT GGCTAGATTG GTTCTTTTTT TTTTTTTTC    6240

CTTTTTTAAG ACGGAGTCTC ACTCTGTCAC TAGGCTGGAG TGCAGTGGCG CAATCAACCT    6300

CCAACCCCCT GGTTCAAGAG ATTCTCCTGC CTCAGCCTCC CAAGTAGCTG GGACTACAGG    6360

TGCACACCAC CATGCCAGGC TAATTTTTGT AATTTTAGTA GAGATGGGGT TTCATCGTGT    6420

TGGCCAGGAT GGTCTCTCCT GACCTCACGA TCCGCCCACC TCGGCCTCCC AAAGTGCTGG    6480

GATTACAGGT GTGAGCCAGG GCACCAGGCT TAGATGTGGC TCTTTGGGGA GATAATTTTG    6540

TCCAGAGACC TTTCTAACGT ATTCATGCCT TGTATTTGTA CAGCATTAAT CTGGTAATTG    6600

ATTATTTTAA TGTAACCTTG CTAAAGGAGT GATTTCTATT TCCTTTCTTA AAGAGGAGGA    6660

ACAAGAAGAT GAGGAAGAAA TCGATGTTGT TTCTGTGGAA AAGAGGCAGG CTCCTGGCAA    6720

AAGGTCAGAG TCTGGATCAC CTTCTGCTGG AGGCCACAGC AAACCTCCTC ACAGCCCACT    6780

GGTCCTCAAG AGGTGCCACG TCTCCACACA TCAGCACAAC TACGCAGCGC CTCCCTCCAC    6840
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TCGGAAGGAC TATCCTGCTG CCAAGAGGGT CAAGTTGGAC AGTGTCAGAG TCCTGAGACA      6900

GATCAGCAAC AACCGAAAAT GCACCAGCCC CAGGTCCTCG GACACCGAGG AGAATGTCAA      6960

GAGGCGAACA CACAACGTCT TGGAGCGCCA GAGGAGGAAC GAGCTAAAAC GGAGCTTTTT      7020

TGCCCTGCGT GACCAGATCC CGGAGTTGGA AAACAATGAA AAGGCCCCCA AGGTAGTTAT      7080

CCTTAAAAAA GCCACAGCAT ACATCCTGTC CGTCCAAGCA GAGGAGCAAA AGCTCATTTC      7140

TGAAGAGGAC TTGTTGCGGA AACGACGAGA ACAGTTGAAA CACAAACTTG AACAGCTACG      7200

GAACTCTTGT GCGTAAGGAA AAGTAAGGAA AACGATTCCT TCTAACAGAA ATGTCCTGAG      7260

CAATCACCTA TGAACTTGTT TCAAATGCAT GATCAAATGC AACCTCACAA CCTTGGCTGA      7320

GTCTTGAGAC TGAAAGATTT AGCCATAATG TAAACTGCCT CAAATTGGAC TTTGGGCATA      7380

AAAGAACTTT TTTATGCTTA CCATCTTTTT TTTTTCTTTA ACAGATTTGT ATTTAAGAAT      7440

TGTTTTTAAA AAATTTTAAG ATTTACACAA TGTTTCTCTG TAAATATTGC CATTAAATGT      7500

AAATAACTTT AATAAAACGT TTATAGCAGT TACACAGAAT TTCAATCCTA GTATATAGTA      7560

CCTAGTATTA TAGGTACTAT AAACCCTAAT TTTTTTATT TAAGTACATT TTGCTTTTTA       7620

AAGTTGATTT TTTTCTATTG TTTTTAGAAA AAATAAAATA ACTGGCAAAT ATATCATTGA      7680

GCCAAATCTT AAGTTGTGAA TGTTTTGTTT CGTTCTTCC CCCTCCCAAC CACCACCATC       7740

CCTGTTTGTT TTCATCAATT GCCCCTTCAG AGGGCGGTCT TAAGAAAGGC AAGAGTTTTC      7800

CTCTGTTGAA ATGGGTCTGG GGGCCTTAAG GTCTTTAAGT TCTTGGAGGT TCTAAGATGC      7860

TTCCTGGAGA CTATGATAAC AGCCAGAGTT GACAGTTAGA AGGAATGGCA GAAGGCAGGT      7920

GAGAAGGTGA GAGGTAGGCA AAGGAGATAC AAGAGGTCAA AGGTAGCAGT TAAGTACACA      7980

AAGAGGCATA AGGACTGGGG AGTTGGGAGG AAGGTGAGGA AGAAACTCCT GTTACTTTAG      8040

TTAACCAGTG CCAGTCCCCT GCTCACTCCA AACCCAGGAA TT                        8082
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(2) INFORMATION FOR SEQ ID NO:12:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 4480 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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TCGTGTCTTG CCCTGTCTTG TCTTGTCTTG TCCTGTGCGC GTGTTCAGTT CGTCTCAGTT    720

TTGGACTCAG ATCTGGGTTT TGGTCGAAGG AGAAGGCCCA GGGCTTCGGT TTCTCAGGGT    780

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TCCTAGCGGG TCTCCGAGGG GCGGCACGAC GCCCCACCAA TTTGGCTCAG GTAAAACAAG    2700

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TGAGTGGAAA GACTCTGAAA TGGGGCTTTC GGACAGTTG ACTTGGACAA GGTTACCACA    4140

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(2) INFORMATION FOR SEQ ID NO:13:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 565 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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TGCCTTATTA GGAAGGCAAC AGACGGGTCT GACATGGATT GGACGAACCA CCGAATTCCG    420

CATTGCAGAG ATATTGTATT TAAGTGCCTA GCTCGATACA ATAAACGCCA TTTGACCATT    480

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(2) INFORMATION FOR SEQ ID NO:14:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 1804 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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ATACAGAAGG AGACTGGTGG GAAGCAAGAT CAATCACTAC AGGAAAGAAT GGTTATATCC    240

TGAGCAGTTA TGTAGCGCCT GCAGATTCCA TTCAGGCAGA AGAATGGTAT TTTGGCAAAA    300

TGGGGAGAAA AGATGCTGAA AGATTACTTC TGAATCCTGG AAATTAATGA GGTATTTTCT    360

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TCCGTCTAAA AAACAAATAA GCAAACAGAA CAAAACAAAA CAAAAACGAG AGAGCGAAAC        720

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AGGTACAATG ATGCCAGAAG CTTTTCTTCA AGAAGCTCAG GTAATGAAAA AAATAAGACA       1140

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CTTCACTGCT ACAGAGCCAT AGTACCAGCC AGGAGAAAAC TTCTAATTCA AGTAGCCTAT       1800

TTTA                                                                   1804
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Claims

1.   A cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, which cellular immunogen comprises host cells which have been transfected with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

2. An immunogen according to claim 1 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

3. An immunogen according to claim 2 wherein the transfected cells are non-dividing.

4. An immunogen according to claim 2 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

5. An immunogen according to claim 4 wherein the mutant DNA is nontransforming.

6. An immunogen according to claim 5 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

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7. A cellular immunogen according to claim 6 wherein the host cells have been transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

8. An immunogen according to claim 1 wherein the host cells have been transfected with a transgene cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

9. An immunogen according to claim 1 wherein the cells comprise fibroblasts.

10. A method for preparing a cellular immunogen for immunizing a host against the effects of the product of a target proto-oncogene, the overexpression of which target proto-oncogene is associated with a cancer, the method comprising:

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of the transgene in the transfected cells, the transgene encoding a gene product which induces host immunoreactivity to host self-determinants of the product of the target proto-oncogene gene.

11. A method according to claim 11 wherein the transgene comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species different from the host species.

12. A method according to claim 11 wherein the transfected cells are non-dividing.

13. A method according to claim 11 wherein the transgene comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

14. A method according to claim 13 wherein the mutant DNA is nontransforming.

15. A method according to claim 14 wherein the mutant DNA comprises a deletion mutation in a region of said DNA which is essential for transformation.

16. A method according to claim 15 wherein the host cells are transfected with a plurality of transgene constructs, each construct encoding a different deletion mutation.

17. A method according to claim 11 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-erbB-2, MDM-2, c-myc, c-myb, c-ras, c-src and c-yes.

18. A method according to claim 1 wherein the excised cells comprise fibroblasts.

19. A method of vaccinating a host against disease associated with the overexpression of a target proto-oncogene comprising
     (a) excising cells from the host;
     (b) transfecting the excised cells with at least one transgene construct comprising at least one transgene cognate to the target proto-oncogene and a strong promoter to drive the expression of

the transgene in the transfected cells, the
transgene encoding a gene product which induces
host immunoreactivity to host self-determinants of
the product of the target proto-oncogene gene;

(c) returning the excised cells transfected
with the transgene construct to the body of the
host to obtain expression of the transgene in the
host.

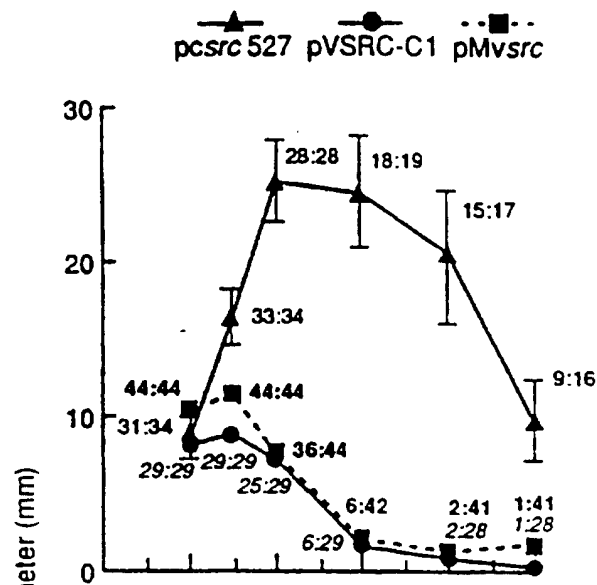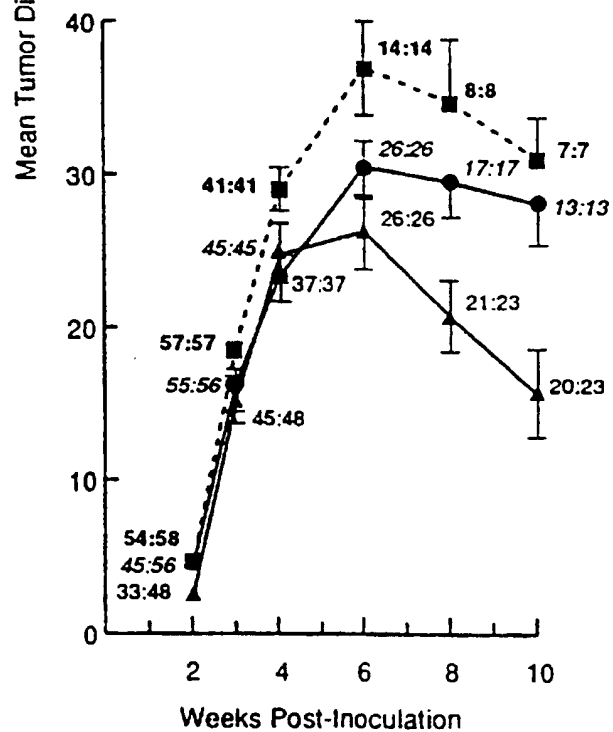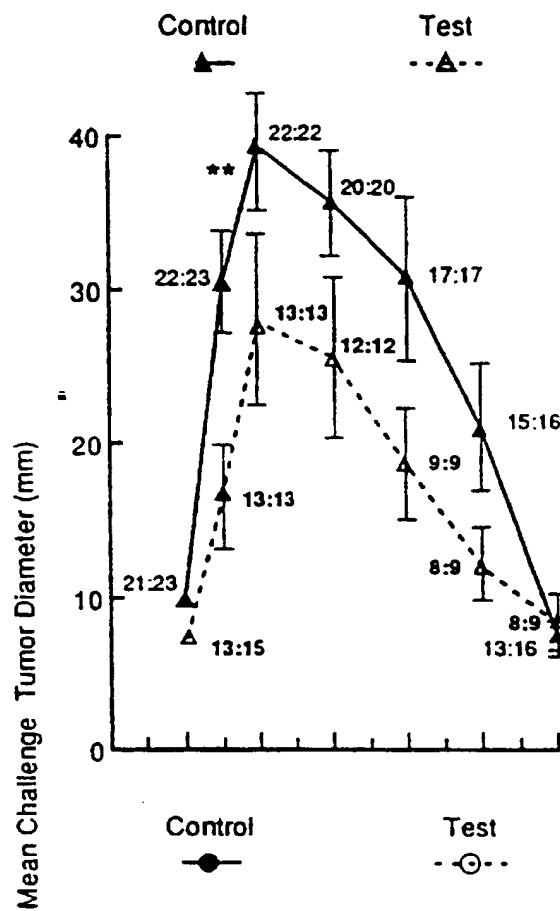20. A method according to claim 19 wherein the transgene
comprises

wild-type or mutant retroviral oncogene DNA; or

wild-type or mutant proto-oncogene DNA of a species
different from the host species.

21. A method according to claim 20 wherein the transfected cells
are rendered non-dividing prior to return to the body of the host.

22. A method according to claim 20 wherein the transgene
comprises mutant retroviral oncogene DNA or mutant proto-oncogene DNA.

23. A method according to claim 22 wherein the mutant DNA
is nontransforming.

24. A method according to claim 23 wherein the mutant DNA
comprises a deletion mutation in a region of said DNA which is essential for
transformation.

25. A method according to claim 24 wherein the host cells are
transfected with a plurality of transgene constructs, each construct encoding a
different deletion mutation.

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26. A method according to claim 19 wherein the transgene is cognate to a target proto-oncogene selected from the group of proto-oncogenes consisting of AKT-2, c-*erbB*-2, MDM-2, c-*myc*, c-*myb*, c-*ras*, c-*src* and c-*yes*.

27. A method according to claim 19 wherein the excised host cells comprise fibroblasts.

28. A method of vaccinating a host against disease associated with the overexpression of a targeted proto-oncogene comprising

(a) excising cells from the host;

(b) transfecting the excised cells with at least one transgene construct comprising at least transgene and a strong promoter to drive the expression of the transgene in the transfected cells, wherein the transgene comprises

(1) wild-type or mutant cognate retroviral oncogene DNA; or

(2) wild-type or mutant cognate proto-oncogene DNA of a species different from the host species;

(c) returning the excised cells transfected with the transgene construct to the body of the host to obtain expression of the transgene in the host.

FIG. 1A

FIG. 1B

FIG. 2A

FIG. 2B

FIG. 3A

FIG. 3B

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01K 63/00; A61K 39/00, 39/38, 48/00; C12N 5/00, 15/00

US CL : 424/93.21, 184.1; 435/172.1, 240.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.21, 184.1; 435/172.1, 240.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPAT, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS

Search terms: oncogene/transfection/vaccine/proto-oncogene

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MALONE et al. Cationic liposome-mediated RNA transfection. Proc. Natl. Acad. Sci. USA. August 1989, Vol. 86, pages 6077-6081, see entire document	1-18
Y,E	US 5,593,972 (WEINER et al.) 14 January 1997, see entire document.	1-28
Y	FENDLY et al. The extracellular domain of HER2/neu is a potential immunogen for active specific immunotherapy of breast cancer. J. Biol. Response Mod. October 1990, Vol. 9, No. 5, pages 449-455, see entire document.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 MARCH 1997	1 2 MAY 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	RAY F. EBERT
Facsimile No. (703) 305-3230	Telephone No. (703) 3058-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FELGNER et al. Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA. November 1987, Vol. 84, pages 7413-7417, see entire document.	1-28
Y	FENTON et al. Cytotoxic T-cell response and In Vivo protection against tumor cells harboring activated ras proto-oncogenes. J. Natl. Cancer Inst. 18 August 1993, Vol. 85, No. 16, pages 1294-1302, see entire document.	1-28
Y	MCCABE et al. Minimal determinant expressed by a recombinant vaccinia virus elicits therapeutic antitumor cytolytic T lymphocyte responses. Cancer Res. 15 April 1995, Vol. 55, pages 1741-1747, see entire document.	1-28
Y	TEMIN, H.M. Overview of biological effects of addition of DNA molecules to cells. J. Med. Virol. May 1990, Vol. 31, pages 13-17, see entire document.	1-28
Y	CONRY et al. Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res. 01 April 1995, Vol. 55, pages 1397-1400, see entire document.	1-28